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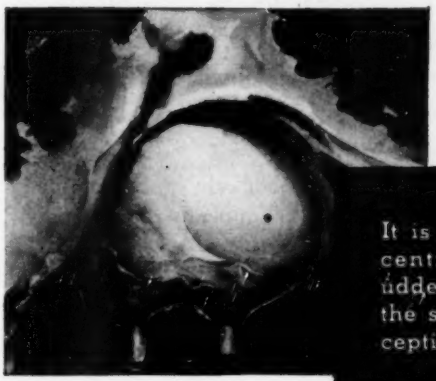

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VOL. X JULY, 1949 No. 36

The Sulkowitch Test as a Guide in the Diagnosis and Therapy of Bovine Hypocalcemia

D. K. DETWEILER, V.M.D., M.S., and J. E. MARTIN, V.M.D.

Philadelphia, Pennsylvania

AN ANALYSIS of the results of serum calcium determinations on bovine blood samples, when correlated with the clinical data which accompanied the samples, revealed occasional errors in diagnosis. This emphasized the urgent need for a simple, rapid method of estimating the serum calcium level suitable for use under field conditions. The veterinary clinician is at a considerable disadvantage in dealing with cases of bovine hypocalcemia since, ordinarily, he is guided only by symptoms and the response of the subject to the administration of calcium preparations. Owing to the inadequacy of present methods, an incorrect diagnosis of milk fever is sometimes made. This may result in the intravenous administration of calcium solutions to animals with normal serum calcium levels. Furthermore, clinicians often want to satisfy themselves regarding the serum calcium level in perplexing cases, even though clear-cut symptoms of hypocalcemia are not present.

In casting about for a test to fill this need, it was found that Sulkowitch[1] had devised a method for rapidly estimating the amount of calcium in the urine. This test was employed by Barney and Sulkowitch[1] in studying human patients with urinary calculi. Albright[2] advocated the use of the Sulkowitch test as a rough estimate of the serum calcium level in human hypoparathyroid patients receiving dihydrotachysterol. Since there is a rough correlation between the level of serum calcium and the amount of calcium being excreted in the urine, it was decided that this test might fill the present need.

From the School of Veterinary Medicine, University of Pennsylvania, Philadelphia.

We wish to express our appreciation to Drs. William B. Boucher and Walter E. LaGrange for supplying blood and urine samples from the clinical cases herein reported, and we thank the following students for their technical assistance: J. A. Himes and Paul Magura.

In this investigation, the authors made all of their determinations in ml.; 1 ml. is equal to 1.000027 cc.

THE SULKOWITCH TEST

Reagent.—The reagent is an oxalate buffer mixture of the following composition:

Oxalic acid	2.5 Gm.
Ammonium oxalate	2.5 Gm.
Glacial acetic acid	5.0 cc.
Water, to make	150.0 cc.

This is a colorless solution with a pH of about 2.3. It is stable and may be kept at least seven months without significant pH change. Its action, when mixed with equal parts of urine, is to precipitate calcium as insoluble calcium oxalate. Bovine urine samples ranging from pH 5.4 to 8.5, when mixed with equal parts of the reagent, produce a solution with a pH range of 2.6 to 4.5. At this pH range, calcium oxalate is precipitated and the alkaline earth phosphates are soluble.

Technique of Test.—The following method has been found most satisfactory when testing bovine urine.

1) Prepare a standard, by adding 5.0 cc. of distilled water to an equal quantity of urine being tested and mix well.

2) In a second test tube of the same diameter, mix equal parts (5.0 cc.) of urine and Sulkowitch reagent.

3) Compare the two test tubes in transmitted light in two minutes and again in ten minutes.

The preparation of a standard, as described, is necessary, owing to the variation in color and turbidity of different urine samples. Typical results of Sulkowitch tests on urine samples containing varying amounts of calcium are pictured in figure 1. For the purposes of this discussion, the reactions pictured have been classified and given symbols (chart 1).

The following serum calcium levels have frequently been associated with tests showing precipitates similar to those pictured.

Test tube (No.)	Serum calcium (mg./100 cc.)
1	below 8.5
2	8.5 to 9.5
3	9.5 to 13.0
4	13.0 to 18.0
5	19.0 and above

EXPERIMENTAL USE OF THE SULKOWITCH TEST

In order to determine the possible usefulness of this test in cattle practice, more than 100 urine samples have been tested. Of these, 63 tests have been correlated with serum calcium determinations. Data obtained from determinations made on the urine and blood serum of 27 different cows are included in this report. Some of these animals had milk fever, some were clinically healthy, and others were suffering from various diseases. Five cows were tested at intervals for various periods from a few weeks up to a year or so. Three acute experiments were performed on 2 cows in which the serum calcium levels were altered by the injection of calcium gluconate or sodium oxalate.

Milk Fever.—The urine from 6 cows with milk fever gave a negative Sulkowitch test. The urine of 3 of these cows was tested a second time, following the intravenous injection of calcium gluconate solution and, in each case, a heavy precipitate (++++) was produced. The serum calcium levels in 3 of these animals, prior to treatment, were 4.2, 4.5, and 7.1 mg./100 cc., respectively.

Normal Cows.—The urine from 10 clinically normal cows was tested. There were 9 adult Guernseys and 1 adult grade cow in the group. Two of these tests were negative at serum calcium levels of 8.2 and 9.2 mg./100 cc., respectively. Five showed a slight precipitate (+) at serum calcium levels from 9.1 to 9.8 mg./100 cc.; and 3 showed an easily detectable precipitate (++) at serum calcium levels from 8.9 to 11.9 mg./100 cc. These results indicate that negative reactions can occur in clinically healthy animals and that the amount of precipitate is not always directly proportional to the serum calcium level. However, in general, the amount of precipitate increases as the serum calcium level rises.

Cows Tested at Intervals.—Serum calcium determinations and Sulkowitch tests were made at various intervals on samples from 5 cows. One animal had a serum calcium level of 9.2 mg./100 cc., thirty-six hours after calving. Three days later, signs suggestive of mild milk fever developed (recumbency, rapid respiration). At this time, the serum calcium level was 7.4 mg./100 cc. and the Sulkowitch test negative. No treatment was given. Five hours later, the animal had improved and was able to stand. At this time, the serum calcium level was 7.5 mg./100 cc. and the Sulkowitch test positive (+). Fifteen hours later, the serum calcium had risen to 8.6 mg./100 cc. and the Sulkowitch test remained positive (+). Recovery was uneventful, the serum calcium level remained above 8.4 mg./100 cc., and the Sulkowitch test continued to show the presence of a small amount of calcium in the urine (+). About ten months later, the serum calcium level was 10.0 mg./100 cc. and the Sulkowitch test positive (++).

Samples were taken from two cows (nonpregnant) six times at varying intervals over periods of eleven and fourteen months, respectively. The serum calcium levels ranged from 9.1 to 11.3 mg./100 cc. and the Sulkowitch reactions were positive (+ to ++). The smaller quantities of precipi-

Chart 1—Symbols Used to Classify Reactions

Test tube (No.)	Symbol	Reaction	Description of precipitate
1	—	Negative	No visible precipitate
2	+	Positive	A slight precipitate visible as a fine white mist when compared with standard
3	++	Positive (moderate)	An easily detectable precipitate varying from a fine white cloud to a somewhat opaque haze
4	+++	Positive (marked)	A distinct precipitate, more or less milky in appearance
5	++++	Positive (very heavy)	A very heavy, chalky precipitate which settles out rapidly

The above symbols are used in the accompanying tables to indicate the degree of precipitate obtained.

tate (+) were associated with serum calcium levels below 10.0 mg./100 cc.

Samples were taken from a fourth cow four times over a six weeks' period. The serum calcium level ranged from 8.5 to 10.7 mg./100 cc. The Sulkowitch test was positive (+), in each instance, except the time when the serum calcium level was 8.5 mg./100 cc.

Two sets of samples were taken two weeks apart from a fifth cow, which was between four- and five-months pregnant. The serum calcium levels were 8.5 and 9.2 mg./100 cc. and the Sulkowitch test was negative both times.

From the foregoing data, it is clear that there is rough correlation between the serum calcium level and the presence of detectable amounts of calcium in the urine. However, a negative Sulkowitch test may occur when the serum calcium is in the low normal to normal range. The normal range, as found by Allcroft,[7] is from 8.5 to 11.4 mg./100 cc.

Results of Acute Experiments.—Three experiments were performed on 2 cows in order to determine the character of the Sulkowitch test at widely different serum calcium levels. Another purpose was to determine the serum calcium level at which calcium begins to appear in the urine in sufficient quantity to be detected by the Sulkowitch reagent.

In these experiments, a sterile 20 per cent solution of calcium gluconate, containing 4 per cent of boric acid, was injected intravenously to raise the serum calcium level. A sterile 3 per cent sodium oxalate solution was administered intravenously to lower the serum calcium level. The injections were made by gravity flow or with a 100-cc. syringe into the jugular or mammary veins. Blood samples were taken from any of the jugular or mammary veins not used for injection purposes. The bladder was drained of urine a few minutes before each sample was taken, and the sample tested with the Sulkowitch reagent consisted mostly of urine elaborated during a three- to four-minute period just prior to collection. A metal catheter was inserted into the bladder to obtain the urine samples and allowed to remain in place. Both of the subjects strained frequently during the experiments

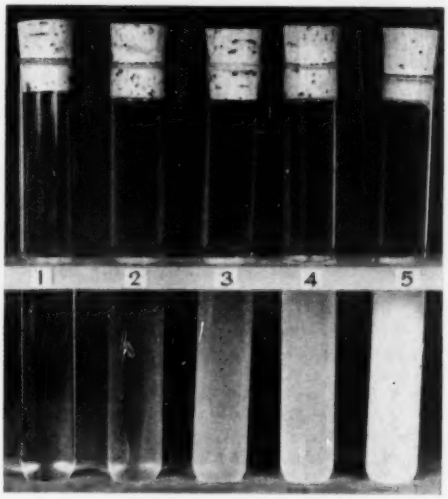

Fig. 1—Typical examples of the Sulkowitch test. See text.

TABLE 1—The Effect of a Rapid Increase in Serum Calcium on the Sulkowitch Test (Experiment 1)

Elapsed time (minutes)	Procedure	Serum Ca (mg./100 cc.)	Sulkowitch test	Heart rate	ECG records	Symptoms
0	Control records	9.4	+	67	1. Normal rhythm	
77	Ca gluconate injection started					
85	30 Gm. Ca gluconate			52	2. Normal rhythm	
100	90 Gm. Ca gluconate			45	3. Partial heart block P-R interval prolonged. Wenckebach phenomenon	Dyspnea, pulse irregular (missed beats)
105		19.0				
110			+ + + + extremely heavy	96	4. Normal rhythm	Marked dyspnea, salivation
155				81	5. Normal rhythm	Pulse rapid, regular; dyspnea, somewhat relieved
165		14.9	+ + +			
265				68	6. Normal rhythm	Breathing normally
280		13.2	+ + +			
360				69	7. Normal rhythm	
365		12.3	+ +			

All doses indicated in table are cumulative.

and thus kept the bladder fairly empty at all times. The Sulkowitch tests were performed at the time each urine sample was taken, and the amount of precipitate obtained was used as a guide for the further administration of the drugs. The serum was separated from the blood samples at the conclusion of each experiment and stored overnight in a refrigerator. Serum calcium determinations were made on the following day using the Clark-Collip modification of the Kramer-Tisdall method.[3]

Electrocardiograms were taken at intervals throughout each experiment.

The pertinent data from these experiments are found in tables 1, 2, and 3 and graphically represented in figure 2. The animal used in experiments 1 and 3 was an 875 lb., 8½-year-old Guernsey cow, not pregnant and not lactating. This animal has had an artificial rumen fistula for the past year. The subject used in experiment 2 was a 6-year-old, 825 lb. Guernsey cow, lactating and not pregnant. All doses in the tables are cumulative.

In these experiments, all of the Sulkowitch tests on urine samples, taken when the serum calcium level was 8.2 mg./100 cc. or below, were negative. All urine samples, taken when the serum calcium levels were above 8.3 mg./100 cc., developed a precipitate when the Sulkowitch reagent was added. The precipitate became progressively heavier in the range from 9.7 to 13.2 mg./100 cc., and, at levels above this, the test was milky to chalky in character, becoming heavier as the serum calcium level increased. In experiment 2, a single injection of 3 Gm. of calcium gluconate brought the mildly hypocalcemic serum calcium level to within the normal level and, within five minutes of the injection, the urine, which had previously been negative to the Sulkowitch reagent, showed a positive Sulkowitch reaction. In experiment 3, on the other hand, five, 3-Gm. doses of calcium gluconate, administered over a period of eighty-seven minutes, were required to bring the serum calcium level to the point at which sufficient calcium was passing over into the urine to produce a positive Sulkowitch reaction.

PRACTICAL INTERPRETATION OF THE SULKOWITCH TEST

These studies clearly demonstrate that, in the animals in this series, the amount of calcium in the urine roughly parallels that in the serum, above a certain level. The results of experiments 2 and 3 indicate that the serum calcium level can be estimated, when the Sulkowitch test is negative, by giving repeated test doses of 3 Gm. of calcium gluconate each until urine, elaborated subsequent to the injection, shows a precipitate when mixed with the reagent. However, in different animals, or in the same animal at different times, the amount of

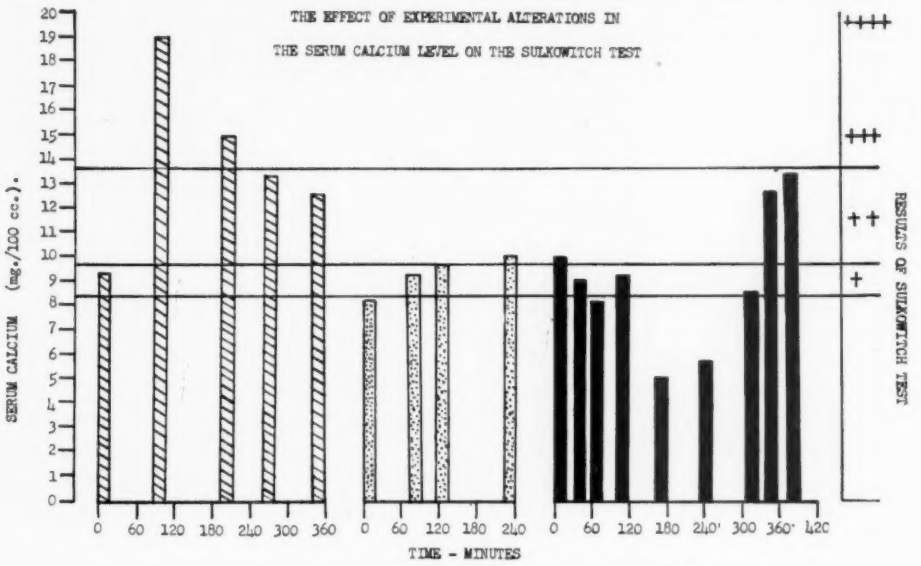
THE EFFECT OF EXPERIMENTAL ALTERATIONS IN
THE SERUM CALCIUM LEVEL ON THE SULKOWITCH TEST
SERUM CALCIUM (mg./100 cc.).
RESULTS OF SULKOWITCH TEST
++++
+++
++
+
0 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
0 60 120 180 240 300 360
0 60 120 180 240
0 60 120 180 240 300 360 420
TIME - MINUTES

Fig. 2—The results of experiments 1, 2, and 3. See text and tables 4, 5, and 6.

precipitate at a given serum calcium level may vary somewhat. There are several reasons for this variation. The amount of precipitate observed will vary with the initial pH of the urine, the time following the addition of the reagent, the intensity of the light, and the visual acuity of the observer. The amount of urinary calcium will vary with the serum calcium level, the diet, and the concentration of the urine. Despite these variables, it appears that this test will be sufficiently informative for most clinical needs under ordinary circumstances. It is needless to point out that, like other tests of this nature, it only has value when correlated with careful clinical observations.

On the basis of the experimental use of this test, the following interpretations are suggested. When the Sulkowitch test is negative (no visible precipitate), the serum calcium level is probably in the low normal range, or below. The calcium level may be estimated by giving repeated test doses of calcium gluconate (3 Gm. each) intravenously and testing the urine elaborated after each successive dose until a positive reaction is obtained. If the precipitate is just detectable or easily seen as a fine white cloud, the serum calcium level is within a satisfactory range. A heavy precipitate, milky or chalky in appearance, indicates that there is danger of hypercalcemia.

In a limited series of diseased animals, there has been found no interference with the test. Samples have been obtained from cows having the following diseases (after each disease, the serum calcium level (mg./100 cc.) and the results of the Sulkowitch test (appropriate symbols) are listed in parentheses): nephrosis with pronounced albuminuria (serum Ca not determined, +); acute mastitis with septicemia (10.8, ++); rickets (8.6, +); osteoporosis (8.2, +); osteoporosis (8.8, ++); metritis, albuminuria (9.8, +); actinomycosis, undergoing x-ray therapy, 4 cases (9.4, +; 9.2, +; 9.7, +; 10.4, ++); acetonemia (serum Ca not determined, ++). Another case in which there was depression and inappetance was not diagnosed (9.0, —). Presumably, renal functional impairment will result in an increased renal threshold for calcium.[4] In man, the injection of parathyroid extract will cause increased urinary calcium which occasionally may occur in the absence of an increased serum calcium level.[4] Shohl[5] has pointed out that, while increases in the excretion of minerals in the urine usually accompany increases in the blood constituents, high urine values may occur with diminished blood levels. In 2 cases which have been brought to our attention by practitioners who submitted blood and urine samples, large amounts of calcum (++++ Sulkowitch reaction) have been detected in the urine despite a normal or low normal serum calcium level.[8,9] Both of these individuals were ill following parturition. Neither case was diagnosed as milk fever. The serum calcium level was 8.3 mg./100 cc. in 1 case and 10.4 mg./100 cc. in the other. Both urine samples contained albumin, and the sample, from the animal with the serum calcium level of 10.4 mg./100 cc., reacted positively to the acetone test. The precipitate from one of these Sulkowitch tests was subjected to spectrographic analysis (courtesy of Dr. D. Crisman, Dairy Laboratories) and calcium identified as the chief cation present. In each case, the char-

TABLE 2—The Effect of Gradually Increasing the Serum Calcium in a Clinical Case of Mild Hypocalcemia* (Experiment 2)

Elapsed time (minutes)	Procedure	Serum Ca (mg./100 cc.)	Sulkowitch test	Heart rate	ECG records	Symptoms
0	Control records	8.20	—	68	1. Normal rhythm	No symptoms of hypocalcemia
40			—			
70	3 Gm. Ca gluconate					
75		9.20	+			
80			+			
85			+			
98	6 Gm. Ca gluconate					
100			+			
110		9.7	+ +			
210	9 Gm. Ca gluconate					
240		10.00	+ +			
245				70	2. No change	

*Allcroft,[7] on the basis of serum calcium determinations on 250 blood samples from cattle has proposed, as a normal range, 8.5 to 11.4 mg./100 cc. Sixty-five per cent of these samples fell within a more narrow range of 9.5 to 10.4 mg./100 cc. and the remaining samples were about equally distributed above and below this range. He defines hypocalcemia as those cases with a serum calcium level of 8.4 mg./100 cc. or lower.

All doses indicated in table are cumulative.

acter of the precipitate was typical of calcium oxalate. Thus, there is no reason to believe that a false positive reaction was involved.

Further use of the test under clinical conditions is required before its limitations can be accurately defined. Wherever possible, the Sulkowitch test should be correlated with subsequent serum calcium determinations. Certainly, it can never be considered more than a substitute for more accurate methods.

TABLE 3—The Effect of Rapidly Lowering and Rapidly Raising the Serum Calcium (Experiment 3)

Elapsed time (minutes)	Procedure	Serum Ca (mg./100 cc.)	Sulkowitch test	Heart rate	ECG records	Symptoms
0	Control records	9.80	+ +	60	1. Normal rhythm	
28	6 Gm. Sodium oxalate					
34		9.00	+	65	2. Normal rhythm Q-T interval slightly prolonged	
42	9 Gm. Sodium oxalate					
48		8.10	—	58	3. Normal rhythm	
75				68	4. Normal rhythm	
100		9.10	+			
125	13.5 Gm. Sodium oxalate					
130			+	70	5. Normal rhythm	
138	16.5 Gm. Sodium oxalate					
143			+			
156	19.5 Gm. Sodium oxalate					
161			+	71	6. Normal rhythm	
168	22.5 Gm. Sodium oxalate					
173				73	7. Normal rhythm	
175		5.0	—	71	8. Normal rhythm Q-T interval further prolonged	
205	25.5 Gm. Sodium oxalate			70	9. Normal rhythm	
245		5.9	—			
260	3 Gm. Calcium gluconate					
265			—			
268	6 Gm. Calcium gluconate					
283			—			
286	9 Gm. Calcium gluconate					
288			—			
308	12 Gm. Calcium gluconate					
318			—			
322	15 Gm. Calcium gluconate					
332		8.3	+			
337	18 Gm. Calcium gluconate					
342			+			
352	42 Gm. Calcium gluconate*			40		Dyspnea, heart rate slowed; pulse irregular
354				40	10. Sino-auricular block P-R interval prolonged Q-T interval shortened. Wenckebach phenomenon	
360		12.4	+ +			
367	58 Gm. Calcium gluconate					
380		13.2	+ +	72	11. Normal rhythm Q-T & P-R intervals the same as in ECG No. 1	

*24 Gm. of calcium gluconate injected rapidly (2 minutes).
All doses indicated in table are cumulative.

THE USE OF THE SULKOWITCH TEST

As a guide to therapy, the Sulkowitch test should make it possible to determine the amount of calcium, injected intravenously, required to overcome a given hypocalcemia. Since the amount of calcium ordinarily required to do this is but a fraction of the dose commonly employed, the dangerous practice of excessive intravenous doses can be avoided. Once the serum calcium level is brought within a satisfactory range, the calcium required to maintain the animal, until normal calcium metabolism is restored, may be supplied by the safer subcutaneous route. It has been found experimentally,[6] and in practice, that this route produces a more sustained, although markedly lower, increase in serum calcium level than the intravenous route. Furthermore, it should be possible to avoid the intravenous use of calcium solutions in animals not suffering from hypocalcemia.

As a guide in diagnosis, this test should aid in differentiating hypocalcemia, hypomagnesemia, and ketosis, and it should serve in demonstrating the coexistence of two or more of these conditions. Moreover, since such diseases as traumatic gastritis, metritis, acute mastitis, and pneumonia may simulate hypocalcemia in the initial stages, or be complicated by hypocalcemia, it should be of value in determining the need for calcium in many cases.

OTHER USES OF THE SULKOWITCH REAGENT

The Sulkowitch test has been used in human medicine to detect increased urinary calcium in cases of urinary calculi and as a guide to parathyroid therapy. It may find similar uses in veterinary medicine. Since calcium metabolism is similar in all domestic mammals, it should be of equal value in the management of hypocalcemia in other species, as well as the bovine.

It is probable that somewhat more reproducible results may be obtained with this test by first adjusting the reaction of the urine so that it is neutral to litmus paper prior to the addition of the Sulkowitch reagent. However, this is not essential and it would complicate the procedure to some extent. There is need for further study of the test under clinical conditions. However, it is useful in its present form and hence this report is published. It is our feeling that further investigation will be better accomplished by those engaged more directly in clinical work than ourselves.

SUMMARY

1) A simple test, suitable for estimating the serum calcium level in cattle, is described.

2) Data obtained from the clinical and experimental use of this test are presented.

3) It is concluded that this test should be of value as a guide in the diagnosis and therapy of bovine hypocalcemia.

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Studies on Repeated Vaccination of Cattle with *Brucella Abortus* Strain 19

I. The Agglutinin Response of Animals Vaccinated as Calves and Revaccinated as Young Adults

D. T. BERMAN, B.A., D.V.M., M.S., and B. A. BEACH, D.V.M.

Madison, Wisconsin

FIELD TRIALS[1,2,3] and controlled experiments [1,4,5,6] have demonstrated that the majority of cows, which had been vaccinated as calves with *Brucella abortus* strain 19, were protected from infection when exposed during the first pregnancy. Although little evidence is available at present concerning the duration of this acquired resistance beyond the first pregnancy, evidence of a definite decrease with time has come from experiments previously reported.[5,7] Reports of declining resistance in vaccinated animals have also come from the field.

The apparently successful bolstering of resistance by repeated immunizations with such agents as typhoid-paratyphoid vaccine suggested that a similar improvement might result from repeated vaccinations with strain 19. Indeed, this possibility has been discussed[8] and the practice is being followed to some extent in Great Britain.[9] Actually, there have been no controlled experiments to study this immunization procedure, other than an observation[10] that guinea pigs which received three small doses of strain 19 had at least as much resistance and possibly more, when exposed to a virulent strain, as those which received a single large dose.

The experiments described here were designed to test the possibilities of repeated vaccinations in cattle. The present report will describe only the agglutinin response of the animals in an experimental herd from the time of vaccination through the termination of the first pregnancy and the outcome of that pregnancy.

MATERIALS AND METHODS

In November, 1945, 64 grade Holstein-Friesian heifer calves, negative to the agglutination test, were purchased on the open market. When the animals were approximately 8 months old, they were vaccinated subcutaneously with 5 cc. of *Br. abortus* strain 19 suspension, which had been freshly prepared in this laboratory according to U.S. Bureau of Animal Industry standards. The calves were then assigned at random to three groups of equal size. The heifers in group 1 were not treated again. Those in group 2 were revaccinated subcutaneously six months later with 5 cc. of fresh strain 19 vaccine, when the animals were approximately 14 months old. The animals in group 3 were similarly revaccinated twelve months after the first vaccination, or when they were approximately 20 months old. The three groups mingled as a unit thereafter and made up herd 5.

The animals were bred by natural service when they were 15 to 18 months old, and no attention was paid to the time of vaccination, so that many of the animals in group 3 were in the first trimester of pregnancy when vaccinated for the second time.

All animals were blood tested at least at monthly intervals and, more frequently, immediately after vaccination. End titers of the serums were determined by means of the conventional tube test. At each parturition, portions of the placenta and quarter samples of colostrum were cultured on thiamin-enriched tryptose agar plates, containing 1 unit of penicillin[11] and 0.0125 mg. of sodium azide[12] per cubic centimeter of medium, to inhibit possible contaminants. Preliminary experiments indicated that this medium was not inhibitory to small inoculums of several strains of *Br. abortus*, and it has been successfully employed in making isolations from badly contaminated infectious materials. All cultures were incubated in an atmosphere of CO_2 (10%) at 37 C., and examined every other day for a week. Suspected colonies were tested with known Brucella antiserums.

Portions of placenta also were triturated with sterile sand and the resulting suspensions inoculated into 3 guinea pigs, which were sacrificed and cultured four to five weeks after inoculation. Fetal liver, spleen, and contents of the fetal stomach of dead calves were also cultured, as described above, and inoculated into guinea pigs.

Daily blood cultures, for ten days following

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the second vaccination, were made from 7 animals in group 2 and 10 in group 3. Approximately, 15 cc. of blood was collected from the jugular vein directly into an evacuated, rubber stoppered, 110-cc. serum bottle containing 50 cc. of thiamin-enriched tryptose dextrose broth with heparin as an anticoagulant. These broth cultures were subcultured on tryptose agar plates after seven and fourteen days of incubation at 37 C. No positive isolations were made in this series. Occasional isolations of strain 19 from the blood, using this procedure, have been made in another series of animals which will be described elsewhere.

EXPERIMENTAL RESULTS

The duration of the first gestation periods, fate of the calves, and results of cultures are given in table 1. *Brucella abortus* was isolated from cow 27 in group 2 and cow 20 in group 3, which aborted, and from 2 cows (8 and 64) in group 1. The details of this natural occurrence of brucellosis follow.

On the regular monthly herd test in July, 1947, increases in the agglutinin titer were observed in animals 20 and 27 from incomplete at 1:50 and at 1:25, respectively, on the preceding two months, to incomplete at 1:200 in each case. This was seven and ten months, respectively, after the second vaccination. Both animals were removed from the herd and placed in an isolation pen on the same farm. On the next monthly test, both animals reacted at 1:400, and shortly thereafter, cow 27 delivered a weak calf 231 days after service and retained her placenta, and cow 20 aborted a 231-day fetus.

Brucella abortus was isolated from both placentas, from the aborted fetus, and from all quarter samples of colostrum from both cows. The colostrum whey samples from both cows reacted at 1:400, which was the highest dilution tested. The strain isolated did not require CO_2 for isolation, was virulent for guinea pigs, and grew well in concentrations of thionine blue inhibitory to strain 19.[13] Using the criteria of dye tolerance and virulence for guinea pigs, it was concluded that the infection was not due to strain 19. The animals were slaughtered, and the same strain of *Br. abortus* was isolated from the spleen and supramammary and hepatic lymph nodes of both animals.

TABLE 1—Results of First Pregnancy After Vaccination

Group 1 single vaccination				Group 2 revaccinated after 6 mo.				Group 3 revaccinated after 12 mo.			
Cow (No.)	Duration pregnancy (days)	Calf L or D[1]	Brucella recovered	Cow (No.)	Duration pregnancy (days)	Calf L or D[1]	Brucella recovered	Cow (No.)	Duration pregnancy (days)	Calf L or D[1]	Brucella recovered
2	272	L	No	3	284	L	No	1	280	L	No
8	*	..	Yes	6	280	L	No	4	Not pregnant	(26)[2]	...
11	279	L	No	12	281	L-W	No	7	Not pregnant	(26)[2]	...
16	286	L	No	13	Not pregnant	(19)[2]	...	10	281	L	No
19	280	L	No	15	283	L	No	18	277	L	No
21	274	L	No	22	274	L	No	20	231*	D	Yes
26	284	L	No	23	277	L	No	24	278	L	No
31	281	L	No	27	231*	L-W	Yes	25	282	L	No
33GC	292	L	No	30	283	L	No	28	278	L	No
11ZC	276	L	No	32	287	D	No	29	280	L	No
63	275	L	No	12ZC	278	L	No	35	287	L	No
64	*	..	Yes	11GC	285	L	No	9ZC	Not pregnant	(26)[2]	...
65	274	L	No	20ZC	212*	D	No	1FC	275	L	No
68	283	L	No	62	275	L	No	35ZC	276	L	No
70	277	L	No	66	284	L	No	69	277	L	No
72	277	L	No	67	271	L	No	76	271	L	No
75	Not pregnant	(24)[2]	...	71	276	L	No	78	Not pregnant	(24)[2]	...
79	Not pregnant	(23)[2]	...	73	286	L	No	81	Slaughtered actinomycosis	(16)[2]	...
84	Not pregnant	(16)[2]	No	74	281	L	No	83	284	L	No
				77	Not pregnant	(16)[2]	...	85	270	L	No
				80	Not pregnant	(24)[2]	...	86	283	L	No
				82	281	L	No				
Mean duration pregnancy (days)		279			274				275		

See text. [1]L = calf living; LW = calf living but weak; D = calf dead. [2]The number in parentheses refers to the months after first vaccination animal was kept in the herd, and is a part of the test results.

On the regular monthly herd test in September, 1947, a rise in titer was observed in cow 64 from negative at 1:25, during the past eight months, to 1:40. This occurred sixteen months after the animal's only vaccination, and when she was in the third trimester of pregnancy. The cow was immediately placed in the isolation pen and observed for two more months, during which her titer did not fall. She was slaughtered at 275 days of pregnancy. Cultures of placenta, uterine fluids, and fetal materials were negative, as were cultures of various lymph nodes, spleen, liver, lung, and kidney. Parallel guinea-pig inoculations were all negative except for the animals inoculated with placenta, from which *Br. abortus*, identical with the strain described above (from cows 20 and 27), was isolated.

A rise in titer was observed on the regular herd test in October, 1947, for cow 8, after she had been negative for eight months. This was twenty months after her only vaccination, and in the second trimester of pregnancy. The animal was slaughtered, and a strain of *Br. abortus*, identical with the strain described above, was isolated on culture from the placenta, uterine fluids, fetal tissues, a number of lymph nodes, the liver, and spleen.

Cow 20ZC aborted a 212-day fetus in October, 1947, twelve months after her second vaccination. She was negative to the agglutination test at the time, and remained so thereafter. Cultures and guinea-pig inoculations were all negative. The colostrum whey titer was 1:200, but cultures were all negative for Brucella. Repeated whey agglutination tests were negative at a dilution of 1:12.5, and repeated milk cultures were negative. It was felt that the abortion was not related to the infection described above, nor was there evidence of trichomoniasis or *Vibrio foetus* infection. The animal was, accordingly, retained in the herd and bred for her second pregnancy with the others.

At the present time (10 months after the last infected cow was removed from the herd), there has been no further sign of infection in this group of animals. Unfortunately, there were no unvaccinated animals in the herd at that time, so it is impossible to estimate the extent to which all of the heifers were exposed. It has not been possible to determine the source of infection.

THE AGGLUTININ RESPONSE

Figure 1 is a composite curve showing the average agglutinin responses of the three groups from the second month through the twenty-fourth month after the first vaccination, when the first pregnancy had been terminated in all but a few cases. As indicated in the curve, the agglutinin titers receded gradually after the first vaccination, and mean titers were below 1:25 at the time of the second vaccination at either 14 or 20 months of age.

As shown in the curve, there was a rapid anamnestic response to the second vaccination in groups 2 and 3, which was apparent by the second day after the inoculation and which reached its peak, in most cases, on the seventh day. The average peak titer in both groups was about 1:1,600, and the individual maximum was 1:12,800. By the ninth day after the second vaccination, the recession in titer was apparent; and, on the average, the decline was a smooth one, requiring about four months

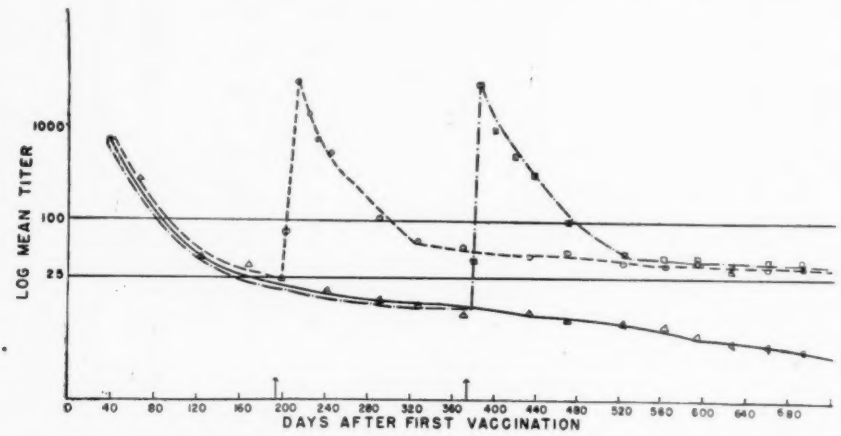

Fig. 1—The average agglutinin response to repeated vaccination with strain 19. Groups 1, 2, and 3 are represented by the continuous line, the broken line, and the line broken by dots, respectively. The time of revaccination is shown by the arrows.

to fall below 1:100 in each of the revaccinated groups. Shortly after falling below 1:100, the average curves for both groups leveled off in the range between 1:25 and 1:100, and have fluctuated between these limits into the second year after the first vaccination. The trend of the level portions of the reaction curves for the revaccinated animals is essentially the same as that for the single vaccination group, but one or two dilutions higher. At twenty-four months after the first vaccination, the average titer in each of the revaccinated groups is about 1:25.

The distribution of negative (negative to incomplete agglutination at 1:25), suspicious (complete agglutination at 1:25 to incomplete agglutination at 1:100), and reactor (complete agglutination at 1:100 or higher) animals in each of the groups from the fifth through the fourteenth months after the first vaccination is given in figure 2, and for the fifteenth through the twenty-fourth months after the first vaccination in figure 3. The month-by-month recession in titers after the first vaccination may be followed in groups 1 and 3 in the first chart. Thus, six months after the first vaccination, there were 4 negative and 15 suspect cows in the first group; and 8 negative, 11 suspect, and 2 reactor animals in group 3. In the eleventh month after the first vaccination, just before revaccination in group 3, the distribution was 12 negative and 3 suspect cows in group 1, and 17 negative and 3 suspect animals and 1 reactor in group 3. Animal 9ZC retained a 1:400 titer for the year following her first vaccination. This rose to 1:1,600 after revaccination, returned to 1:400 within two months, and remained at that level through the next twelve months. As shown in table 1, this cow failed to conceive.

In the sixth and seventh months after the first vaccination, the animals in group 2 received their second dose of strain 19 vaccine. This is reflected in figure 2 in the large number of reactors in that group in the seventh and eighth months. The same type of response may be seen in figure 2 following revaccination in group 3 in the twelfth month after the first vaccination.

The charts also indicate that in each revaccinated group the distribution of reactor, suspect, and negative animals was more or less stabilized within three to five months after the second vaccination. This also is reflected in the level portions of the average curves in figure 1. For example, in the eleventh month, five months after their second vaccination, there were 4 reactor, 16 suspect, and 2 negative animals in group 2. In the twelfth month, five months after their revaccination, the distribution in group 3 was 2, 13, and 6. In the twenty-fourth month, eighteen months after the animals in group 2 were revaccinated and a year after revaccination of group 3, the distribution was 10 negative and 8 suspect cows and 1 reactor in group 2, and 9 negative and 9 suspect animals and 1 reactor in group 3. This is in contrast with the distribution in the single vaccination group, which was 14 negative, 1 suspect, and no reactors at this time.

Examination of the month-to-month reactions to the test of the individuals in the revac-

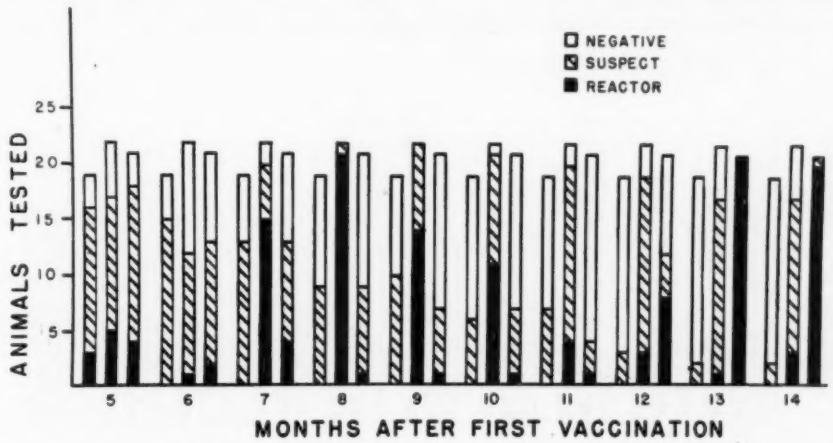

Fig. 2—The distribution of reactor, suspect, and negative cows from the fifth through the fourteenth months after the first vaccination. For each month, the first bar represents group 1, the second bar group 2, and the third bar group 3.

cinated groups reveals considerable fluctuation within the limits of the suspect class. That is, from month to month, an individual animal's titer might vary from 1:25 to 1:50. Occasionally, a negative animal showed a temporary reaction of 1:25 or 1:50, and from time to time, a suspect might show a titer of 1:100 for a month or two.

DISCUSSION

The relatively rapid recession of titers following revaccination in the animals described in this report is of considerable theoretic as well as practical interest. Studies of the relationship between the age at the time of Brucella vaccination and the persistence of agglutinins have clearly shown that the titers of most animals vaccinated as adults persist for a considerably longer period than is the case with animals vaccinated as calves.[14] Revaccination of adult animals, reacting to strain 19, results in an increase in agglutinin titer,[15] similar to that described here, but no data are shown on a subsequent decline in agglutinins.

Calves born to infected dams show a lower and less persistent agglutination reaction, following vaccination with strain 19, than those born to uninfected dams.[16] The workers were not able to duplicate this result by early (10 to 100 days of age) vaccination and revaccination at 1 year of age.

The results of the present work show a striking similarity to the so-called "Koch phenomenon" in tuberculosis, a manifestation of infection immunity. There is a more rapid and greater development of agglutinins following revaccination than is seen after a first vaccination, but there is also a much more rapid recession of the agglutinin titer than is observed in animals vaccinated for the first time at ages of 14 or 20 months. This might be attributable to the resistance engendered by the first vaccination partially interfering with the persistence of the second dose of strain 19, and thus leading to a rapid fall in the agglutinin titer. If this should be the case, it would be interesting to know for how long a period following the first vaccination animals will remain in a state of partial resistance to further inoculations with strain 19. Unpublished data from another herd indicate that, for some animals at least, the period may be several years in length.

It remains to be seen whether repeated vaccinations with strain 19 will produce any increment of resistance to virulent infection over that engendered by a single vaccination. Unfortunately, we have no knowledge of the source or extent of exposure to the virulent strain from which the several cases of infection were apparently derived, nor of the influence that such probable exposure may have on the future response of the animals remaining in the herd. Any interpretations of the efficacy of repeated vaccination in this herd will have to be qualified by the possible influence of the exposure to virulent infection.

SUMMARY

Two groups of 22 and 21 calves were vaccinated with *Brucella abortus* strain 19 at approximately 8 months of age and revac-

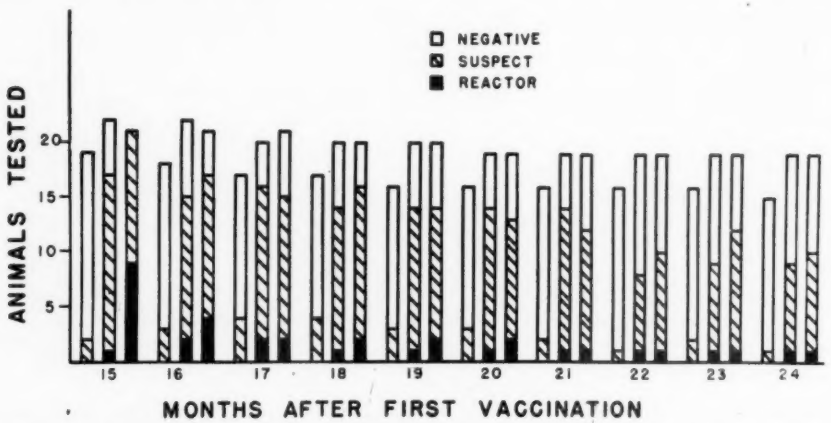

Fig. 3—The distribution of reactor, suspect, and negative cows from the fifteenth through the twenty-fourth months after the first vaccination.

cinated at either 14 or 20 months of age. Following revaccination, there was a rapid rise in the agglutinin titer, which then fell, on the average, below 1:100 within three months and was about 1:25 at the termination of the first pregnancy. At this time, there was 1 reactor in each group.

These results were compared with those from a group of animals vaccinated only once, and they showed the typical return to a negative status within twelve months. A spontaneous outbreak of virulent infection occurred in the experimental herd, which will have to be considered in any future evaluation of the efficacy of revaccination with strain 19 in this herd. Revaccination did not appear to have any deleterious effect on the first pregnancy of the animals.

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The Use of Two Antimalarial Substances in the Treatment of Anaplasmosis

HERMAN FARLEY, D.V.M., M.S., C. C. PEARSON, D.V.M., LON E. FOOTE, D.V.M., and I. O. KLIEWER, A.D.

Stillwater, Oklahoma

CATTLE ANAPLASMOSIS is taking $4,000,000 to $6,000,000 annually out of stockmen's pockets. In Oklahoma alone, the losses may amount to $1,000,000, or more, per year.

Anaplasmosis is certainly a world problem, although little is known of the extent of the disease in many countries. It has been diagnosed in South Africa, German East Africa, German West Africa, Argentina, Italy, Java, Russia, the Philippine Islands, and other countries. It has also been diagnosed in 29 states in the United States, and is especially serious in the Midwest, South, and Southwest. Active cases have been found in most of Oklahoma's 77 counties, since Stiles[5] diagnosed the first case near Ponca City in 1927.

It is a baffling disease. Research workers have been studying it continuously, but relatively little is known about diagnosis, treatment, or prevention.

REVIEW OF LITERATURE

Anaplasmosis has been regarded as a specific disease of cattle that is caused by the protozoan parasite *Anaplasma marginale*, since it was first diagnosed by Theiler[6] in 1910. Darlington,[3] in 1926, reported what was probably the first case of pure anaplasmosis in the United States. He referred to the disease as having existed in southeastern Kansas for several years. Because it is accepted that a protozoan parasite is the causative factor, drugs, suspected of having therapeutic value in the treatment of protozoan diseases, have been used extensively in treating cattle known to be infected with anaplasmosis.

The effects of antimalarial drugs on avian malaria have been studied extensively since 1945. Curd, Davy, and Rose[2] synthesized antimalarial substances and subsequently developed biguanide. Two of the substances developed from biguanide were found to have a pronounced action on the blood parasites of certain types of avian malaria, and there was a pronounced action on exoerythrocytic forms. Paludrine 4888 was found to stand alone in the intensity and range of its activity. These workers were able to study the action of the antimalarial substance against developmental forms of Plasmodium as it occurred in several species of Plasmodium in birds. Because of their success in treatment of bird malaria, these workers suggested that paludrine 4888 be tried as a prophylactic and therapeutic agent in the treatment of different types of human malaria.

Adams, Maegraith, *et al.*[1] investigated the therapeutic action of paludrine 4888 on acute cases of benign tertian malaria. They reported a clinical cure of 147 cases of benign tertian malaria, and that there were no serious side effects following oral administration of paludrine 4888 in doses ranging from 12 mg. to 700 mg. at twelve-hour intervals for fourteen to twenty-eight days.

These workers were fortunate in that it was possible to study the action of drugs on exoerythrocytic forms and the other developmental forms of Plasmodium organisms. Developmental forms of *A. marginale* have never been observed when studying blood tissue of cattle affected with bovine anaplasmosis. Neither has a developmental cycle been demonstrated in artificial culture mediums or within the body tissues of the known biologic vectors, which are ticks, flies, and mosquitoes. Therefore, it is necessary to study the course of the disease in cattle from a clinical laboratory standpoint, with special reference to changes in the blood picture throughout the course of the disease.

MATERIALS AND METHODS

Osage County, in northeastern Oklahoma, includes some of the best cattle-grazing land in the Southwest, and now has a cattle population of nearly 100,000. Anaplasmosis has been a menace to the cattle industry in that area since the disease was first reported in the state in 1928. During the summer and fall of 1941 and again in 1943, the disease increased greatly in Osage County and in other parts of the state. As cattle prices advanced, the economic loss became enormous. Largely because of this situation, cattlemen in Osage County and other sections of the state were

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instrumental in getting the legislature to make a special appropriation of $100,000 with which the Oklahoma A. & M. College Experiment Station could organize the Oklahoma Veterinary Research Institute. The Institute was founded for the purpose of studying cattle anaplasmosis and other livestock diseases in the state.

The college at Stillwater is the headquarters for the Institute. The administrative offices and two laboratories are located in a campus building. A large brick and cement barn, located 1 mile west of the campus, is equipped with water, lights, floor drains, feed rooms, screened stalls, and other space for cattle, sheep, and small animals. A laboratory has been installed in the east end of the building. Screening tests with drugs and animals are conducted here before being released for more extensive study at other laboratories in and near Pawhuska, Osage County.

A 910-acre ranch, 5 miles southeast of Pawhuska, was purchased by the Institute. It has good fences, two barns, corrals, chutes, a ranch house, water, and lights. Cattle are kept on this ranch under the same general conditions as are found on other ranches in the area. A large, completely equipped laboratory was established in a brick building in downtown Pawhuska.

Another unit at Pawhuska includes a leased barn and 35 acres of good grazing land. There are four screened stalls with cement floors, water and electricity, corrals, loading chute, squeeze chute and stanchion, and a field laboratory in one end of the building. It is at this unit that most of the large-scale therapeutic studies of active cases of anaplasmosis are conducted.

Ranchers in Osage County have been coöperative in furnishing necessary information on herds, history of outbreaks, and other useful information.

The Institute has studied approximately 20 different drugs and combinations of drugs during the past two years. However, only two of them will be discussed in this report. They are paludrine hydrochloride and quinoline diphosphate.

EXPERIMENTAL PROCEDURE

In order to assure accuracy in determining the presence of beginning anaplasmosis in cattle inoculated at the field laboratory, three preinjection observations were made at one-day intervals, including temperature, pulse, respiration, hemoglobin determination, r.b.c., and to determine whether or not Anaplasma-like bodies were present. This precautionary measure is necessary in order to avoid the error made by some individuals who observe a few bodies in r.b.c.'s that they cannot differentiate from *A. marginale* and thereby administer a drug and claim a cure, when the animal in question did not have anaplasmosis originally. It is easy to make a mistake of this kind when trying to check the progress of the disease under range conditions. Beginning ten days after inoculation of the cattle, clinical observations were made at one- and two-day intervals, and blood smears were examined from each animal. When Anaplasma-like bodies were observed in sufficient numbers to determine beginning developmental stages of anaplasmosis, it was considered the minimum time to begin treatment with oral administration of paludrine. Other animals were allowed to advance further in infection, in order to study the effects of the antimalarial substance on Anaplasma and other pathologic changes in the blood picture that might possibly show a deviation different from that in the nontreated animal. With the definite lack of knowledge of the behavior of *A. marginale*, it is not possible to evaluate the efficiency of chemotherapy in anaplasmosis without observing the blood picture throughout the course of the disease. Each of sixteen cattle, 3 to 8 years of age, were treated orally and intravenously with 0.2-Gm. to 4-Gm. doses of paludrine hydrochloride over a period of two to seven days. The average normal r.b.c. at the beginning of treatment, the percentage of Anaplasma-infected blood, r.b.c., and the lowest reading of r.b.c. for each animal were recorded during the course of the disease. Although complete records were recorded for each animal, it was not convenient to submit this voluminous material for publication. With one exception, it was found that if the animal was treated before more than 15 per cent of the erythrocytes showed typical Anaplasma bodies there was recovery.

Approximately the same type of information as that recorded for oral administration of paludrine was included for intravenous injection of the soluble form of paludrine. However, treated cattle apparently respond more readily following this form of treatment. In one instance, an active case, a 9-year-old cow was not treated until 25 per cent of the erythrocytes showed typical Anaplasma bodies and the animal died.

Maegraith, Adams, *et al.*[4] conducted similar tests with paludrine 4888 in treating acute benign tertian malaria. They found that patients could withstand from 50 mg. hourly (for 12 hr.) to 600 mg. hourly for fourteen days without serious side effects.

Doses of 2 Gm. to 4 Gm. of paludrine were administered intravenously in 24- to 48-hour intervals. The 4-Gm. dose was apparently the most satisfactory.

The antimalarial quinoline diphosphate*

*7-chloro-4-(4'-diethylamino-1'-methylbutylamino)-quinoline diphosphate.

was used intravenously in treating active cases of anaplasmosis that were produced at the laboratory. Doses varying from 200 mg. to 2,000 mg. were administered at 24- and 48-hour intervals. The 400-mg. dose was thought to produce the best results. The animals varied in age from 2 to 8 years. No losses were observed in the cattle 2 years of age. It was noted that older cattle apparently had just as fine a chance to recover following treatment as those 3 and 4 years of age.

Quinoline diphosphate was administered intravenously to field cases of acute anaplasmosis in 400-mg. to 2,000-mg. doses at 48-hour intervals. The same records were made of field cases as were made of laboratory cases, with the exception that only one preinjection observation was made. Also, it was not convenient to make 24- and 48-hour observations in all field cases; however, this was done in approximately 50 per cent of the cases. The recovery rate was not in favor of the younger age groups, as one would ordinarily expect.

Most field cases of the disease were not called to our attention until the r.b.c.'s had dropped below 3,000,000 per cubic millimeter.

All laboratory cases that were treated with paludrine and quinoline diphosphate were checked by animal inoculation to determine whether or not carrier infection was destroyed. It was not convenient to check for carrier infection in each individual animal. They were tested in groups of 5 and 10 cattle.

The antimalarial drugs used in this report have shown more promise than any drug that we have used to date. The cycle of infection was apparently inhibited when these drugs were administered, and approximately 85 per cent of all cattle treated for anaplasmosis, under field and laboratory conditions, made a recovery. It is not suggested that the antimalarial drugs as used in these studies will be the decisive therapeutic agents in successfully controlling active cases of anaplasmosis, but there are definite responses in actively infected animals, following the administration of these drugs. Additional anaplasmosis-infected cattle are being treated experimentally with paludrine and quinoline diphosphate.

CONCLUSIONS

1) Anaplasmosis probably takes an annual toll of $4,000,000 to $6,000,000 from the cattle population in the United States.

2) It was first diagnosed as a specific disease of cattle by Theiler in 1910.

3) Investigators have found antimalarial substances to be effective against several species of avian Plasmodium and acute benign tertian malaria in man.

4) Anaplasmosis has continued to progress over the nation until it is now recognized in at least 29 states.

5) Paludrine and quinoline diphosphate have been effective in combating anaplasmosis in Oklahoma cattle.

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Renal Excretion Following Intravenous Injection of Calcium Salts in the Normal Cow

A. H. CRAIGE, JR., V.M.D., R. B. JOHNSON, A.B., E. G. BLACKBURN, and J. M. COFFIN, V.M.D.

College Park, Maryland

THE PROMPT response of the cow with milk fever to intravenous injection of calcium salts suggests a sudden reversal in the physiologic processes responsible for the milk fever state. Since most cases are much improved within two or three hours after treatment, it may be assumed that the favorable physiologic reactions occur within that period. An approach to the identification of those reactions has been made through a study of the effects of intravenous injection of calcium salts on urinary excretion in the normal cow.

PROCEDURES

Four normal dairy cows, weighing 735 to 1,180 lb., were used in this study, 3 of which were dry (1, 2, 3) and the other (4), three months in lactation, was producing about 60 lb. of milk daily. Each cow received a single intravenous injection of each of the following solutions: (1) calcium gluconate, 115 Gm., with boric acid, 11.5 Gm., in water to make 500 cc.; (2) calcium chloride ($CaCl_2 \cdot 2H_2O$), 37.7 Gm., in water to make 500 cc. Thus, each cow received duplicate injections of the same dose of calcium (10.5 Gm.) as the borogluconate and as the chloride.

The effects of these injections were studied by observing the physical symptoms and examining specimens of blood and urine collected before, during, and after the period of acute hypercalcemia caused by the injections. Blood samples were taken immediately before beginning the injection, then at three minutes, one hour, and two hours, and again at twenty-four hours after completing the injection. Urine specimens were collected continuously, beginning one to two hours before the injection and continuing through the injection period for two or three hours thereafter, with an additional specimen on the following day. All urine specimens were quantitative, for periods ranging from forty-five minutes to two hours.

Urine specimens were collected by two methods. A rubber bag was suspended from the vulva and emptied at frequent intervals. This method could not be used when the stool was soft, because feces would contaminate the specimen; but it was entirely satisfactory when the stool was well formed. The second method consisted in inserting a rubber catheter in the urethra, anchored by means of an air-filled balloon or a specially dilated tip, and piping the urine to a jar suspended at the side of the stall. This method was the more satisfactory for continuous collection regardless of the feces, but difficulty arose at times in maintaining the catheter in position.

Serum from the blood specimens was examined for calcium following the Tisdall method,[1] and for inorganic phosphorus by the Youngburg procedure.[2] The same methods were used for calcium and phosphorus in the urine. In addition, the urine was subjected to the following determinations: pH (Beckman, model G apparatus), bicarbonate (calculated from CO_2 capacity[3] and pH[4]), chloride (Van Slyke and Sendroy[5]), total organic acids (Van Slyke and Palmer,[6] employing electrometric titration from pH 7 to 2.7), ammonia (Van Slyke and Cullen[7]), creatinine and creatine (Folin[8]), and phenols (Volterra[9]). All results for urine constituents have been expressed as hourly output.

Injection of the calcium salts into the cows was at the rate of 50 cc. per minute (*i.e.*, ten minutes for the complete injection). During the injection period, the head of the cow was held fast, but, at all other times, it was allowed the freedom of its stanchion stall. Hay and

TABLE 1—Urinary Excretion Following Intravenous Calcium Salts

	Calcium gluconate				Calcium chloride			
Cow	1	2	3	4	1	2	3	4
Urine								
pH	–	–	–	–	+	0	–	0
Bicarbonate	–	–	–	–	+	–	–	–
Ammonia	+	+	–	–	0	–	–	–
Calcium	+	+	+	+	+	+	+	+
Phosphorus	0	–	0	0	0	–	0	0
Chloride	+	+	+	+	+	–	+	–
Organic acids	+	+	+	+	–	–	–	–
Creatinine	0	+	–	+	0	0	0	–
Creatine	–	–	–	+	0	0	0	–
Volatile phenols	–	–	–	–	–	–	–	–
Aromatic hydroxy acids	0	–	–	0	0	–	+	–
Residual phenols			–	+	+	–	+	–
Volume	+	+	+	+	+	0	+	–
Blood								
Calcium	+	+	+	+	+	+	+	+
Phosphorus	+	–	+	–	+	–	+	–

Results indicate an increase (+), decrease (–), or no change (0) in the hourly output of the urinary constituent, or in the blood level, during the two- to three-hour period immediately following injection of the calcium salt.

From the Maryland Live Stock Sanitary Laboratory, University of Maryland, College Park.

In this investigation, the authors made all of their determinations in ml.; 1 ml. is equal to 1.000027 cc.

water were accessible throughout these experiments.

RESULTS

Preliminary Period.—During the preliminary period of the eight experiments, the cows appeared entirely normal, eating hay contentedly and excreting urine at rates varying from 4 to 21 cc. per minute (ave. 10 cc.). Preliminary urine specimens were collected over periods ranging from sixty-three to 137 minutes (ave. 102 min.). Urine pH at this time ranged from 7.8 to 8.6 (ave. 8.1). Normal variations in the hourly output of the urine constituents during this period were as follows: bicarbonate, 20 to 91 mEq. (ave. 50); chloride, 1 to 104 mEq. (ave. 42); total organic acid, 20 to 68 mEq. (ave. 43); ammonia, 14 to 118 mg. (ave. 61); calcium, 6 to 109 mg. (ave. 33); creatinine, 209 to 600 mg. (ave. 361); creatine, 216 to 1,120 mg. (ave. 534); volatile phenols, 139 to 575 mg. (ave. 354); aromatic hydroxy acids, 14 to 490 mg. (ave. 184); and residual phenols, 120 to 890 mg. (ave. 328). Phosphorus was virtually absent from the urine (*i.e.*, less than 1 mg. per 100 cc.) in six of the eight experiments, while cow 3 excreted 49 and 65 mg. P per hour, respectively, during the preliminary periods of its experiments.

Calcium Gluconate.—Following the intravenous injection of calcium gluconate, there was a pronounced increase in the rate of urine output and in its acidity. Rate was increased three- to tenfold, to a maximum of 50 cc. per minute, while pH fell to a minimum of 5.6 in one experiment. The peaks of volume and acidity were reached during the first or second hours following injection of the calcium salt, and normal levels were restored within twenty-four hours.

As indicated in table 1, the period following injection of calcium gluconate was characterized by increased urinary excretion of calcium, total organic acids, and chloride, and by reduced output of bicarbonate, creatine, and volatile phenols. Creatinine, ammonia, aromatic hydroxy acids, and residual phenols were not affected consistently in these experiments, and inorganic phosphorus remained totally absent in three experiments and disappeared from the urine of the fourth during the post-injection period. These results are illustrated by the protocol of a typical experiment (table 2).

Coinciding with the above changes in urine composition, there was a marked but transient increase in blood calcium, accompanied by a secondary rise in blood phosphorus in two of the four experiments, with phosphorus reductions in the other two. It is interesting to observe that in the two experiments where there was a secondary rise in blood P, the urine remained free of phosphorus. Serum calcium levels rose from a preliminary average of 10.0 to 23.0 mg. per 100 cc., measured three minutes following completion of the injection, and normal levels were restored within twenty-four hours. Physical symptoms, including rapid respiration, slight tachycardia, inappetence, polyuria, diarrhea, and muscular tremors, usually appeared during, or immediately following, the injection of calcium gluconate.

Most of the physical symptoms, except diarrhea, had abated or entirely disappeared within two hours after the injection, and, at this time, blood calcium had receded to an

TABLE 2—Calcium Gluconate Experiment*

Blood (results expressed as mg. per 100 cc. of serum)					
Time	11:56	12:10	12:58	14:13	10:00*
Calcium	9.8	25.2	22.0	15.1	10.3
Phosphorus	4.5	4.6	3.8	3.7	1.2

Urine (output per hour)†					
Time	10:30-11:51	11:51-12:54	12:54-14:10	14:10-15:45	9:54-11:14‡
Volume, cc.	600	890	1,925	4,000	670
pH	8.5	7.0	7.2	7.5	8.2
Bicarbonate	35	11	25	48	30
Organic acids	43	89	123	96	46
Chloride	0.43	0.50	4.44	7.10	0.32
Ammonia	106	87	113	144	50
Calcium	12	532	485	311	11
Phosphorus	trace	trace	trace	trace	trace
Creatinine	210	210	290	330	450
Creatine	450	530	380	230	290
Volatile phenols	440	300	270	250	380
Aromatic hydroxy acids	150	140	140	140	180
Residual phenols	220	280	260	380	290

*Cow 4, normal, lactating 64 lb. daily; eating hay at beginning of experiment. Injection intravenous; time 11:57-12:07; 500 cc., containing calcium gluconate, 115 Gm.; boric acid, 11.5 Gm.; in water to 500 cc. Physical symptoms: anorexia, slight muscle tremors, diarrhea, transient dyspnea.

†Bicarbonate and organic acids expressed as cc. of N/1 HCl, chloride as Gm. of Cl, other constituents as mg., per hour.

‡Specimens collected on the following day.

average of 13.7 mg. per 100 cc. At no time did the physical symptoms reach a severity to be alarming; in fact, the reaction was mild enough in cow 1 to pass unnoticed by the casual observer.

Calcium Chloride.—A comparable dose of calcium chloride injected in the same manner into the same cows produced more severe symptoms and significantly different results in the urine. In all four experiments, there was an initial reduction in urine volume during the injection period and immediately thereafter, and, in only two experiments, did it later return to and exceed the preliminary rate. Acidity was increased in only one experiment, where the pH fell from 8.5 to 7.9. These differences were reflected in the chemical composition of the urine.

As with the gluconate, the injection of calcium chloride was followed by increased urinary excretion of calcium, but organic acid output was reduced. Excretion of bicarbonate and chloride was increased in two and diminished in the other two experiments, as was the excretion of volatile phenols, but the effects on creatine were less consistent. Also, ammonia output was diminished in three of the experiments. The other results were inconsistent, as indicated in table 1.

As with gluconate, on an average, calcium chloride caused a rise in serum calcium from 10.1 to 25.0 mg. per 100 cc., which, by the end of two hours, had subsided to 16.3 mg. per 100 cc. and, after twenty-four hours, had returned to preliminary levels. Then again, the same 2 cows (1 and 4) showed a secondary rise in blood phosphorus, accompanied by no urinary excretion of phosphorus throughout the experiment. Many of the same physical symptoms were observed following the chloride as with the gluconate, but they were more severe following the chloride. The physical symptoms developed immediately with the gluconate, whereas they were delayed usually fifteen to twenty minutes following calcium chloride, with the cow usually beginning to eat and appearing entirely normal immediately after the injection.

DISCUSSION

In comparing the effects of these two calcium salts, it is interesting to note that calcium gluconate has virtually replaced the originally recommended calcium chloride in veterinary medicine. Undoubtedly, preference for the gluconate has stemmed much from its relative freedom from caustic action. Whether it possesses other advantages, we cannot say, but there are veterinarians who believe calcium chloride to be superior to the gluconate for the treatment of milk fever in cows.

Support for the claim to superiority for calcium chloride may be found in these experiments. Apparently, the injected calcium remained in the blood stream somewhat longer when the chloride was injected, and it was excreted less rapidly in the urine than when an equal dose of the gluconate was injected. If we attribute the beneficial action of the calcium salt to the injected calcium, we can thus explain the superiority of the chloride. Presumably, the more sustained blood calcium levels, following injections of calcium chloride, may be attributed to retarded renal excretion, which, in

TABLE 3—Calcium Chloride Experiment*

Blood (results expressed as mg. per 100 cc. of serum)

Time	12:55	13:10	14:09	15:15	9:30*
Calcium	10.5	25.4	19.1	17.5	11.3
Phosphorus	5.1	3.8	4.5	3.0	7.2

Urine (output per hour)†

Time	11:49-12:52	12:52-14:11	14:11-15:14	15:14-16:45	9:26-10:54‡
Volume, cc.	1,130	990	650	1,060	870
pH	8.2	8.1	8.1	8.3	8.3
Bicarbonate	91	39	30	62	41
Organic acids	58	34	31	31	36
Chloride	3.69	2.63	1.53	1.63	0.39
Ammonia	76	44	51	45	66
Calcium	109	188	101	49	25
Phosphorus	trace	trace	trace	trace	trace
Creatinine	300	180	220	250	250
Creatine	220	300	200	240	530
Volatile phenols	300	200	120	160	360
Aromatic hydroxy acids	140	90	70	80	140
Residual phenols	310	160	180	150	180

*Cow 4, normal, lactating 60 lb. daily; eating hay at beginning of experiment. Injection intravenous; time 12:57-13:07, 500 cc., containing calcium chloride ($CaCl_2 \cdot 2H_2O$), 37.7 Gm.; in water to 500 cc.
Physical symptoms: anorexia, hyperesthesia, salivation, slight cough, dyspnea, profuse diarrhea (feces well formed before injection, became watery).

†Bicarbonate and organic acids expressed as cc. of N/1 HCl, chloride as Gm. of Cl, other constituents as mg., per hour.

‡Specimens collected on the following day.

turn, may be the result of circulatory disturbances associated with the injection. Thus, it seems probable that the advantage of higher blood calcium level following calcium chloride may be overshadowed by the physiologic strain conceivably dangerous to the stricken cow.

It has been established by clinical experience that milk fever can be treated effectively with calcium gluconate, calcium chloride, and other salts of calcium. This implies that the action is not due to any one anion, but it may be the result of either the specific effect of calcium ion or to some other common characteristic of all these calcium salts. The acidifying action of soluble calcium salts has been suggested.[10] In the present study, an attempt was made to determine the effects upon body functions during the period immediately following the injection of calcium salts, which is the period when recovery occurs with this treatment for milk fever.

Studies involving quantitative renal excretion must be interpreted with consideration for the factors which affect renal activity, as well as variations in the amounts of substances available for excretion. It was observed in these experiments that injection of calcium gluconate was followed immediately by increase in urine volume, whereas urine output actually was decreased during the first hour after calcium chloride, and, in three of four experiments, it remained low. Dyspnea, muscular tremors, anxious expression, and diarrhea were noted during the early postinjection period, and these symptoms appeared more pronounced with the chloride than with the gluconate. It is probable that the more severe systemic reaction to calcium chloride indicates a circulatory reaction on the kidney sufficient to counteract the diuretic effect of the injected fluid and salt.

A comparison of the effects of calcium gluconate and calcium chloride on renal excretion brings out differences which are attributed to the anions and therefore probably not directly concerned with the therapeutic action. Calcium gluconate consistently caused a marked drop in urine pH, associated with reduction in bicarbonate and increased excretion of chloride and total organic acids. Calcium chloride, on the other hand, produced no definite change in urine pH, although bicarbonate tended to be reduced, and total organic acid excretion was decreased in all experiments. It is assumed that the difference in total organic acid levels could be attributed partly to the injected borogluconate. Surprisingly, chloride excretion was actually decreased in two of the calcium chloride experiments.

The most consistent effects on urinary excretion common to both calcium gluconate and calcium chloride were an increase in excretion of calcium, presumably clearing the blood of injected calcium, and a reduction in the output of volatile phenols. Less consistent, but worth considering, were the reductions in aromatic hydroxy acids and in creatine. But, the effect on volatile phenols was the most striking in this series of experiments. It is not known whether this effect was due to suppressed production of volatile phenols, to their retention in the tissues, or to their excretion by other channels (*i.e.*, intestines and lungs). Further study is required to determine the fate of volatile phenols and the possibility of a relationship between their production and the pathology of milk fever.

CONCLUSIONS

1) Intravenous injections of calcium gluconate and calcium chloride into normal cows resulted consistently in an immediate rise in the urine output of calcium and a reduction in the output of volatile phenols.

2) Less consistently, these injections were followed by reduced urine excretion of aromatic hydroxy acids, creatine, and bicarbonate.

3) Effects were not consistent on the other urinary components studied: ammonia, chloride, creatinine, residual phenols, and total organic acids.

4) Renal excretion of inorganic phosphorus, virtually absent from the urine of the normal cow, does not appear to play a part in the acid-base adjustments following injections of calcium salts.

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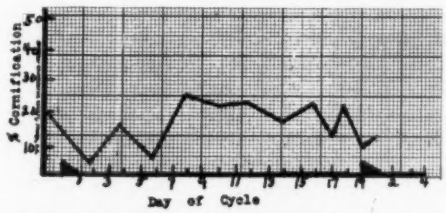
The Vaginal Smear of the Cow and Causes of Its Variation

W. HANSEL, M.S., S.A. ASDELL, M.A., Ph.D., and S. J. ROBERTS, D.V.M., M.S.

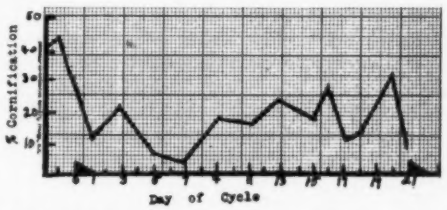
Ithaca, New York

EXPERIENCE WITH the use of hormones as a means of improving the breeding of dairy cattle[1] has led the writers to believe that, before rational hormone therapy can be instituted, much more information is needed on the effects of hormones upon the reproductive tract during the normal cycle and upon the dose levels which will produce these effects. The practitioner has very little on which to rely except rule of thumb results of experience in treatment in the field, for the quantitative aspect of hormone therapy has not yet been placed upon a firm basis. Accordingly, the efforts of this laboratory have been oriented during the past few years to fill in some of the gaps in our knowledge of the normal processes of reproduction in dairy cattle and to establish the responses of the accessory sex organs to various levels of sex steroid hormones and their synthetic substitutes. Others have worked on certain aspects of these problems and a comprehensive general summary has been published.[2] The material here presented concerns mainly the vaginal smear of the cow and the hormonic factors which modify it.



Graph 1—Percentage of cornified cells in vaginal smears of heifers during normal estrous cycles. Triangle represents estrus.

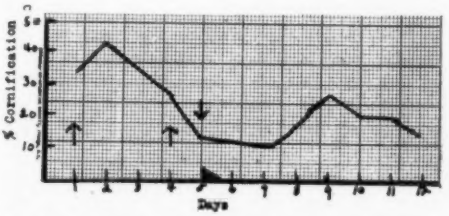
The practical value of the vaginal smear technique is to use it as a means of determining the condition of the internal reproductive organs by a process of correlation. The method works well in rodents and in certain carnivores, e.g., the dog, in which the vaginal smear changes are clear cut and directly correlated with ovarian conditions. In primates, it is not so valuable, for the changes are relative and not absolute, probably due to the high levels of circulating steroid hormones, never entirely absent. The cow, too, lacks clear cut changes in the smear at different stages of the cycle for two reasons, the low hormonic level at all times and the complex nature of the vaginal epithelium.



Graph 2—Percentage of cornified cells in vaginal smears of heifers during normal estrous cycles. Triangle represents estrus.

LITERATURE CITED

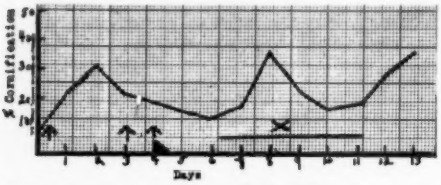
It has been pointed out by Murphey,[3] Hammond,[4] and Cole[5] that the vaginal epithelium in the cow is not uniform throughout the length of that organ. In the region near the os uteri, the epithelium is stratified, but mucus-secreting cells and very thin epithelial cells form the surface layer. As one proceeds toward the vestibule, goblet cells become abundant and then decrease in number. Near the vestibule, there is a region in which the stratified epithelium is very thick, and the superficial layers consist of flattened cells which are cornified to some extent. The structure approaches that of true skin. In this area, many lymph nodes are present. Cyclical changes, which must be present if the vaginal smear is to vary and be of diagnostic value, exist, but



Graph 3—Percentage of cornified cells in the vaginal smear of an ovariectomized heifer injected with diethylstilbestrol. Arrows represent injections. Triangle represents estrus.

Laboratory of Animal Nutrition, New York State College of Agriculture (Hansel and Asdell); and New York College of Veterinary Medicine (Roberts), Cornell University, Ithaca.

they are not clear cut. The authors cited above have described these changes as they were found in material at autopsy. Brown[6] has described them for the upper vagina from biopsy material. All are agreed that the most



Graph 4—Percentage of cornified cells in the vaginal smear of an ovariectomized heifer brought in heat with diethylstilbestrol and then given a series of daily injections of progesterone. Arrows represent diethylstilbestrol injections; triangle, estrus; continuous line, progesterone injections; and X, hemorrhage.

marked cyclic changes occur in the cervical region. In this part of the vagina, the mucous cells are tall and are secreting during proestrus and estrus. At two days postestrus, they are collapsed but the deeper layers are growing. Leucocytes are most abundant at one to two days postestrus. In the vestibular region, the changes are less. Cole describes some cornification at about nine days postestrus. Cole and Hammond have both found an invasion of leucocytes during, and just after, heat. This invasion has ceased at two days but, according to Cole, there is another invasion at nine to eleven days. Most vaginal smears are taken near the cervix, but changes in the vestibular region must be considered since cells shed from this region may work toward the cervix.

Murphey[3] was the first to use vaginal smear techniques in the cow. He inserted a glass rod into the vagina through a speculum, washed it onto a slide with physiologic saline, dried fixed, and stained with hematoxylin and eosin. Zupp[7] continued his work and reported the results of a detailed study almost simultaneously with Frei and Metzger.[8] Cole[5] and Hammond[4] have also described its changes. There are some discrepancies in details. Zupp emphasized the presence of cornified cells during estrus, while Cole and Hammond found relatively few. Hammond recorded an increase in their numbers from five days onward. Frei and Metzger reported the absence of leucocytes at estrus, a finding that Cole was unable to verify. Hammond found small numbers from five days postestrus to the onset of the next heat, while Frei and Metzger reported large numbers at ten to fifteen days postestrus. Hammond found that they were numerous toward the end of heat.

Recently, de Allende, Shorr, and Hartman[9] reported a comparative study of the vaginal smears of the Rhesus monkey and the human being. They used the method of staining introduced by Shorr,[10] a modification of Masson's trichrome stain. This method gives the nucleus and protoplasm of the cells characteristic colors which may be used for differentiating several types which differ in frequency at certain stages of the cycle. This method of staining has been used in our work. Relative frequencies of cell types have been worked out for normal heifers and the most characteristic changes during the cycle noted. This work has been followed by the injection of hormones, diethylstilbestrol, and progesterone into ovariectomized heifers and by the study of changes in the smear under these conditions. This was done in an attempt to find some meaning for the fluctuations observed in the normal heifers.

MATERIALS AND METHODS

In the work with normal heifers, smears were taken at one- or two-day intervals, during at least one complete cycle, from 6 animals —3 Guernseys and 3 Holstein-Friesians. All

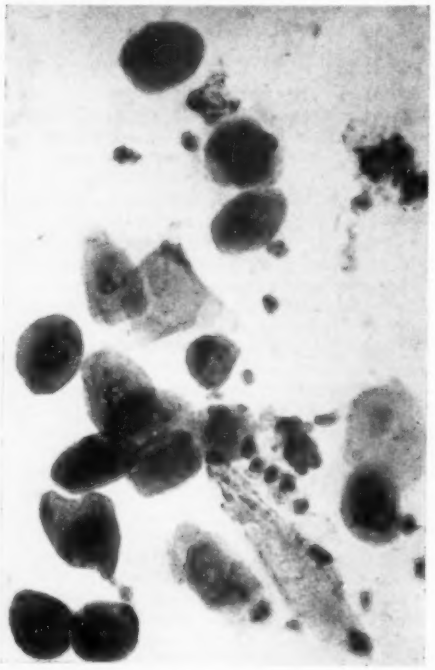


Fig. 1—Vaginal smear of a normal heifer at one day postestrus, showing the predominance of type 2 epithelial cells. Few cornified cells are present. Note leucocytes adhering to the surface of some epithelial cells.

were approximately 3 years of age, in good condition, and were free from infectious diseases. None of them had been bred.

The material for the smears was obtained by means of a pipette and a strong bulb. Saline solution was not used in any case, although, at certain stages of the cycle, it was necessary to make several attempts before

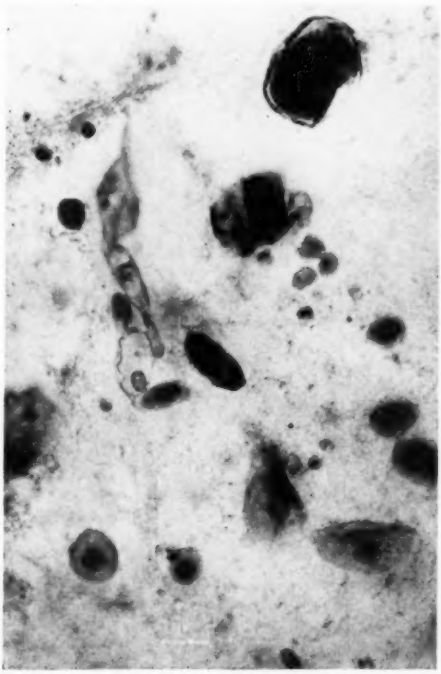


Fig. 2—Vaginal smear of a normal heifer at three days postestrus. Cornified cells are slightly more numerous than at one day postestrus. Very few leucocytes are present.

enough material could be obtained for a smear. In each case, two samples were taken, one from the vagina proper, near the cervix, and one from the vestibular portion. The first smear was invariably taken from the cervical end of the vagina. The vaginal contents were transferred from the pipette to a slide, spread out with the end of the pipette, and immediately fixed in a solution of equal parts of 95 per cent alcohol and ether. It was found essential to spread the smear as thinly as possible before fixation.

After fixation, the slides were stained using Shorr's[9] technique. The method consists essentially of nuclear staining with Harris alum hematoxylin and of cytoplasmic staining the mordanted smears with Biebrich scarlet—orange G—fast green, F.C.F. It was found desirable to treat the smears with chloretone before fixation, when much mucin was present. If this were done, the cells could be seen in greater detail under the microscope. Colorless diaphane was found superior, as a mounting medium, to the damar recommended for the method.

In examining the slides, the presence and relative numbers of leucocytes were noted. Then, at least 500 cells were classified and counted. The cells observed in the bovine smears did not fall entirely into the classification used by de Allende *et al.*, and the following five types proved most suitable for this work.

Type 1.—A small, round cell with a sharp outline. It has a large, centrally placed, darkly staining nucleus and a narrow rim of cytoplasm. This usually stains blue or green, occasionally red. These cells are about 10 to 15 μ in diameter.

Type 2.—A large, roundish cell with a fairly distinct outline which is not always exactly round, but usually has a smooth contour. The nucleus is oval and fairly large but comparatively much smaller than that of type 1 cells. It usually stains purple and contains a well-defined chromatin network. The cytoplasm

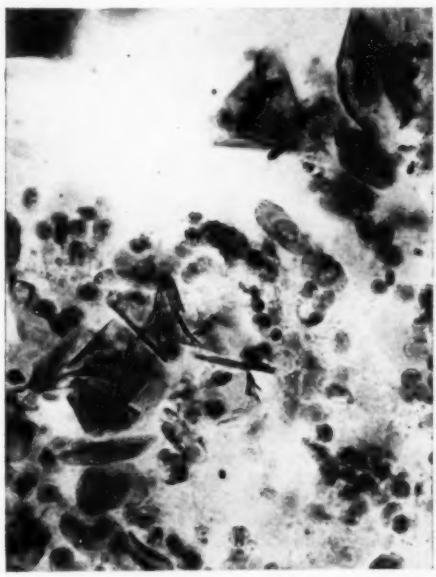


Fig. 3—Vaginal smear of a normal heifer at nine days postestrus. The cornified cells and type 2 epithelial cells are present in considerable numbers. Leucocytes are numerous.

stains a light greenish blue. The edges of this cell show no folding and there are no striations in the cytoplasm. They are for the most part between 18 and 30 μ in diameter.

Type 3.—This type includes all cornified cells. The nuclei are usually eccentric and pyknotic, with an occasional perinuclear halo. The cytoplasm is often finely granular and it stains pink, red, or occasionally yellow. These cells that could be distinctly recognized were classified.

Typical cells of each type are labeled in figures 4 and 10.

Description of the Smears from Normal Heifers

The estrus cycle has been divided into five parts in the discussion which follows: (1)

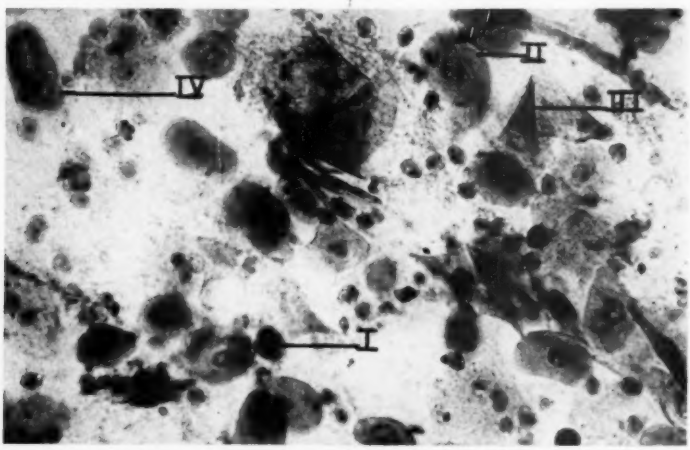


Fig. 4—Vaginal smear of a normal heifer at sixteen days postestrus. The fall in the percentage of cornified cells has not yet occurred in this case. Compare with figure 5, showing a vaginal smear from the same heifer one day later.

cells usually occur in clumps in the smear and they vary greatly in size as well as in shape. These facts make it difficult to count them accurately. The cornified cells appearing in the smears taken late in proestrus differ from the above description in that they are less angular and take the red stain less intensely.

Type 4.—Morphologically, cells of this type resemble type 3, although on the average, they are somewhat larger and the cell outline is less angular. The edges are diffuse and frequently folded. The nuclei may or may not be pyknotic, and they usually stain the same color as the cytoplasm, but more intensely. Purple nuclei are seen occasionally. The cytoplasm stains a pale bluish green and contains striations as well as granules. The cells are usually about 20 μ in the narrowest dimension and 30 μ or more in the longest, although considerable variation exists.

Type 5.—This type includes all cells showing signs of disintegration, such as poorly defined borders and fragmented or absent nuclei. In many cases, they are merely elongated fragments. Very few cells were placed in this class, since, for the most part, only

estrus; (2) one to two days postestrus; (3) two to nine days postestrus; (4) nine to sixteen days postestrus; and (5) sixteen days postestrus to estrus. All animals did not show identical changes in the vaginal smears in each of the above divisions of the cycle. The following discussion applies specifically to the smears taken from the cervical end of the vagina. Those taken near the vestibule show similar changes, but the percentage of cornified cells is persistently higher.

1) Estrus.—In contrast to the findings in other species, smears taken at this time in the cow contained few cornified (type 3) cells. This was apparent in all cases except 1, which was decidedly atypical. In the normal smears, most of the nucleated epithelial cells had striated cytoplasm, folded or diffuse edges, and pyknotic nuclei (type 4 cells). A few cells of types 1 and 2 were present, while leucocytes were numerous (fig. 7).

2) One to Two Days Postestrus.—At this stage, most cells were of the large, round type with purple-staining nuclei (type 2). Type 4 cells were present in fairly large numbers, but type 3 cells were always few (fig. 1). Leuco-

cytes and type 1 cells were scanty. Erythrocytes were found in the smear in several cases on the second day, and large, completely vacuolated cells were occasionally seen.

3) Two to Nine Days Postestrus.—During this period, the percentage of cornified cells was low, except for a slight rise on the third or fourth day (graphs 1 and 2). In the smears of 1 heifer, this rise in cornified cells at the

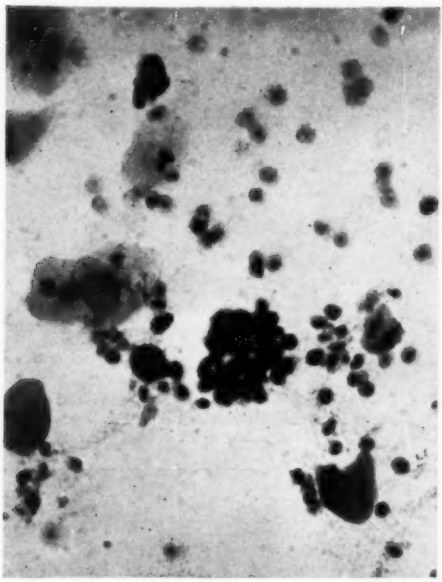


Fig. 5—Vaginal smear of a normal heifer at seventeen days postestrus. Few cornified cells are present. Leucocytes are numerous.

third or fourth day did not occur. Type 4 cells predominated throughout this period and many degenerating cells were found (fig. 2). Type 1 and type 2 cells were practically absent until the seventh or eighth day, at which time they appeared in small numbers, while leucocytes were scanty throughout.

4) Nine to Sixteen Days Postestrus.—At the eighth or ninth day of the cycle, the percentage of cornified cells rose sharply and remained high or continued to rise until the sixteenth or seventeenth day (graphs 1, 2 and fig. 3). In 1 case, however, cornified cells were most plentiful at the eighth day and then declined steadily until the seventeenth day. Type 2 cells were fairly numerous throughout, but they appeared in largest numbers at the fifteenth or sixteenth day (fig. 4). Few type 1 cells were observed during this period. Leucocytes appeared in all the smears, while a few erythrocytes were present in the smear taken from 1 heifer at the eleventh day of the cycle.

5) Sixteen Days Postestrus to Estrus.—In 5 of the 6 heifers studied, the percentage of cornified cells declined sharply at the beginning of this period. Following this decline, their percentage was somewhat variable but it tended to rise until one day postestrus (*see* graphs 1 and 2). During this decline, leucocytes were numerous (fig. 5). Type 2 and type 4 cells predominated until the nineteenth or twentieth day, when large numbers of type

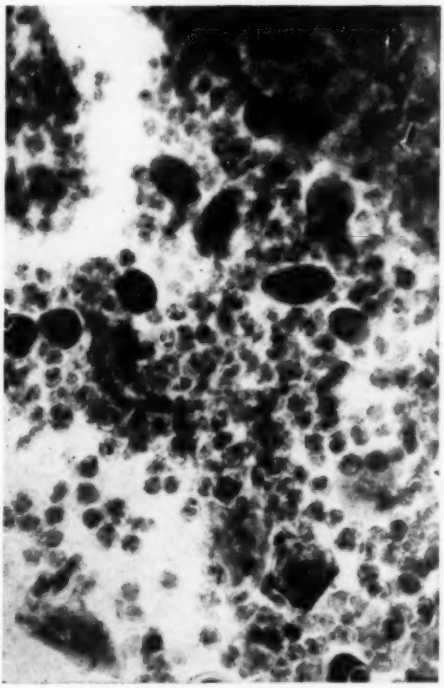


Fig. 6—Vaginal smear of a normal heifer at twenty days postestrus. Most of the small round cells were stained red. Leucocytes are present in very large numbers.

1 cells appeared (fig. 6). Many of the cornified cells (type 3) of this stage seemed to be derived from the type 1 cells, since they were less angular in form and stained a less intense red than those at other stages of the cycle. In 3 of the other heifers, the decline in cornified cells occurred a little later, while in the remaining 1, it had already set in.

THE EFFECTS OF HORMONE INJECTIONS ON THE DISTRIBUTION OF CELL TYPES

The vagina of each ovariectomized heifer

was relatively dry, the smear was scanty, and it was almost impossible to obtain enough to make satisfactory cell counts. The cells present were mostly types 4 and 5; the latter, cell debris, usually predominated. Occasional cells of types 1 and 2 were seen, while the cornified count was variable (fig. 8).

An injection of 0.5 mg. of diethylstilbestrol was made into each of 4 ovariectomized heifers. This caused, on the next day, an increased percentage of cornified cells and the smear was more abundant (fig. 9). On the following day, types 1 and 2 cells appeared in appreciable numbers, as well as many leucocytes. In one case, erythrocytes also appeared in the smear. On the fourth and fifth days, further injections of 0.5 mg. of diethylstilbestrol were made. Heat was manifested at this time and the percentage of cornified cells fell, as it does at this time in normal heifers. At the same time, there was a marked rise in

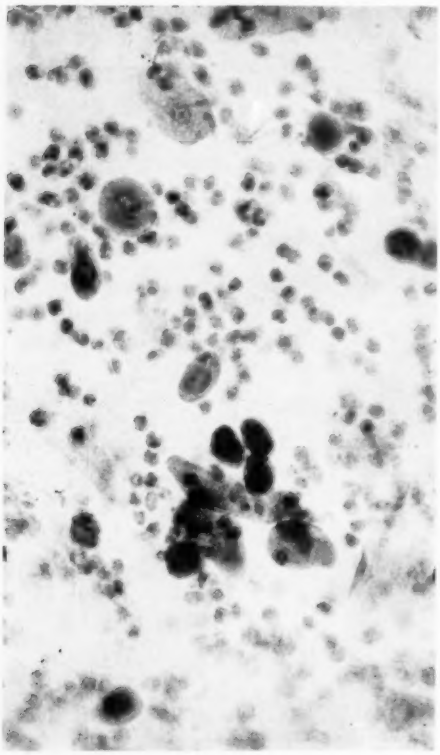


Fig. 7—Vaginal smear of a normal heifer at estrus. Relatively few cornified cells are present. Cell types 1, 2, and 4 are represented. Leucocytes are numerous.

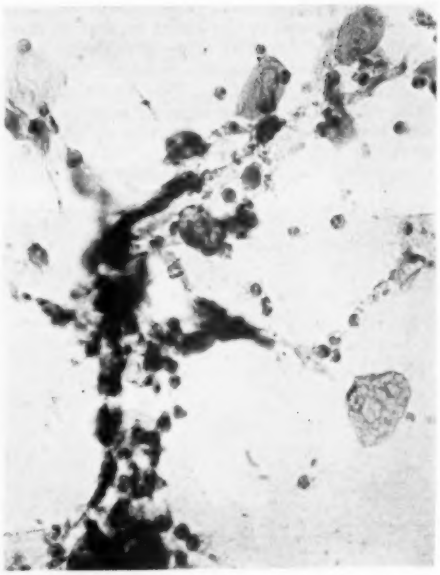


Fig. 8—Vaginal smear from an ovariectomized heifer. The smear consists mostly of cell debris and degenerating cells.

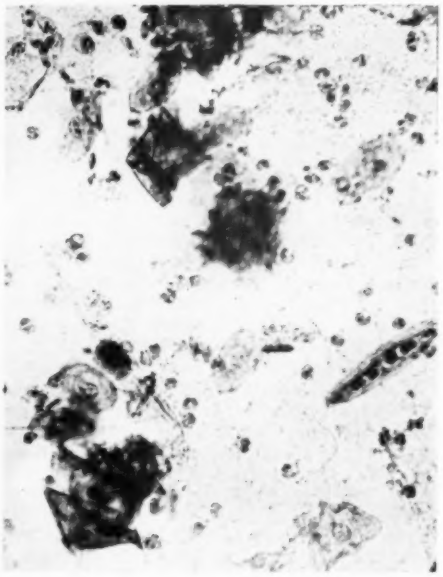


Fig. 9—Vaginal smear from an ovariectomized heifer one day after the injection of 0.5 mg. of diethylstilbestrol. Note the increase in cornified cells.

the proportion of types 1 and 2 cells (fig. 10). Two of these heifers were then followed for several days after they went out of heat. Cell types 1 and 2 decreased while the percentages of types 3 (cornified) and 4 increased, as they tend to do normally after heat (fig. 11). In one of them, prostigmine methyl sulfate was injected but it did not modify the sequence. After a few days, the smear reverted to the ovariectomy type.

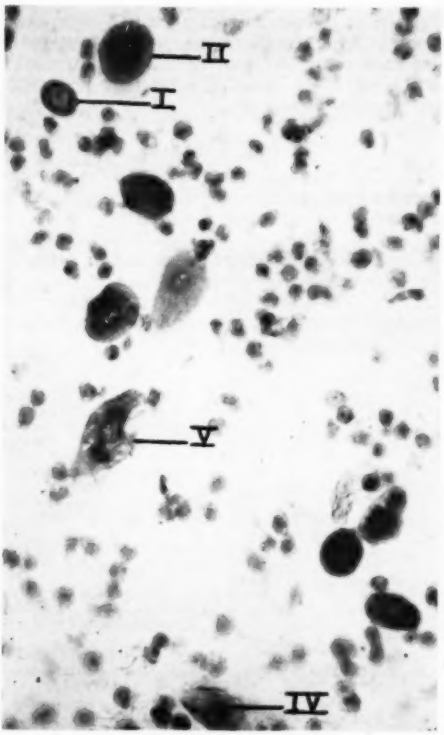


Fig. 10—Vaginal smear from an ovariectomized heifer at diethylstilbestrol-induced estrus. Few cornified cells are present. Cell types 1, 2, and 4 are represented, and leucocytes are numerous. Compare with figure 7, showing the smear of a normal heifer at estrus.

Two of the ovariectomized heifers, which had been brought in heat with diethylstilbestrol, were then injected with progesterone at the rate of 35 mg. daily for six days. The changes in the proportions of cell types followed the sequence described in those heifers which did not receive progesterone, but there was a marked tendency for the level of cornified cells to be maintained (fig. 12). Typical changes in cornified cell distribution are shown in graphs 3 and 4.

DISCUSSION

A study of sections of the vaginal wall stained by Shorr's method indicates that type 4 cells were derived directly from the anterior vaginal wall. The presence of these cells was practically the only distinguishable cell type in the smear of ovariectomized heifers, and the regularity with which they appeared in smears taken throughout the cycle supports this view.

Similarly, type 2 cells appeared to come from the lower layers of the vaginal epithelium and probably to some extent from the mouths of the glands. They were found in the smears in greatest numbers when the epithelium was actively growing, and, again, when it was rapidly reducing.

Type 1 cells appeared to be in various stages of preservation when they were detected in the smears, as indicated by the variety of cytoplasmic-staining reactions which they showed. This fact, as well as their appearance, suggested that they were derived from the cervix or from the uterus.

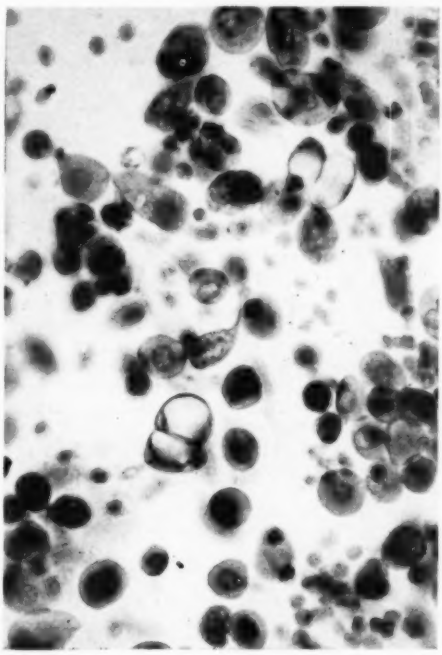


Fig. 11—Vaginal smear from an ovariectomized heifer two days after diethylstilbestrol-induced heat. Cell types 1 and 2 predominate.

The frequency of type 3, cornified cells may be correlated with the stage of the cycle with greater accuracy than any other type. The first response of the vaginal wall to estrogenic stimulation was marked by an increase in the percentage of type 2 cells in the smears, and a correspondingly low percentage of type 3 cells. This growth wave started rather early in the development of

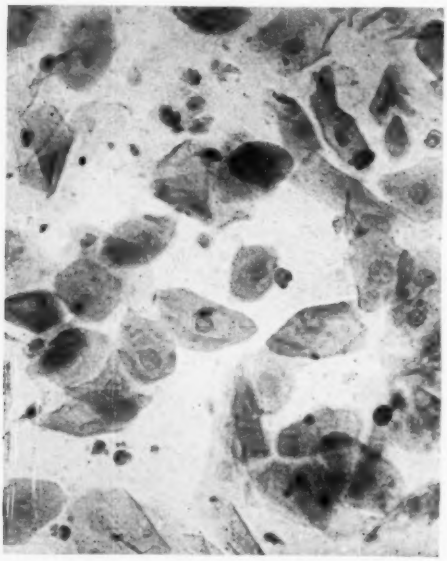


Fig. 12—Vaginal smear of an ovariectomized heifer after diethylstilbestrol and progesterone. Note the presence of large numbers of cornified cells.

the follicle since the cows responded to a low level of estrogens.[11] As growth proceeded, the superficial layers of the vaginal mucosa were farther removed from their blood supply, and, consequently, more of the outermost cells cornified. The growth impulse was never sufficiently great for a large wave of cornification. The percentage of cornified cells did not usually rise above 50 in the cow, in contrast to the 95 per cent which has been reported in the Rhesus monkey.[9] These factors, along with a decreased growth stimulus as the estrogenic effects passed off, resulted in the rise in the percentage of cornified cells from three to five days postestrus. A second growth wave, induced by the reinforcing or growth-maintaining influence of progesterone, resulted in the marked increase in cornified cells from nine to sixteen days. The appearance of tall, mucus-filled cells in the vaginal epithelium and the intense invasion of leucocytes during proestrus resulted in a decrease in all cell types except 1 and 4. This was a period of considerable denudation.

The changes in the smears of ovariectomized heifers supported this interpretation of the smears obtained during the normal cycle. The percentage of cornified cells was greatest when progesterone was injected. It also increased, but not to the same extent, as the effect of injected estrogens wore off (*see* graphs 3 and 4).

SUMMARY

1) The vaginal smears of heifers have been studied, using quantitative methods of estimation of cell type frequency.

2) The effect of ovarian hormones on the vaginal smear of ovariectomized heifers has been similarly studied.

3) The origin of the cell types observed and the causes of their quantitative variation are discussed.

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Virus Papular Dermatitis of the Horse

R. W. McINTYRE, D.V.M.

Los Angeles, California

IN MAY, 1948, the Los Angeles County Livestock Department investigated a condition affecting the skin of Thoroughbreds. This paper presents the case history, clinical symptoms, laboratory examinations, and experimental inoculations which were used in arriving at a diagnosis.

HISTORY

The first case was observed on April 16, 1948, in a 2-year-old filly. Small, concentric, papular swellings appeared in the skin on both sides of the body. Within two weeks, the swellings became generalized except the skin of the head and inner surface of each thigh.

The second case appeared sixteen days later.

The third case developed May 17, and was followed shortly thereafter by 4 others. Most of the lesions in these cases were observed on the left side, *i.e.*, over the shoulder, on the side of the neck, and behind the shoulder girdle.

Six new horses were added to this group on May 12. One of these animals showed skin lesions six days after arriving.

By June 28, or within two months after the beginning of this outbreak, the disease had spread to 17 of the 22 horses in the barn. The majority of these animals were 3-year-olds.

Fig. 1—Primary stage. Right side. Papular dermatitis in a 3-year-old Thoroughbred.

An 8-year-old exercise pony, associated with these animals, had scattered lesions on the right rump, apparently one of the contact points in training the Thoroughbreds. The first lesions on the pony occurred the sixth day following some trial runs with a mare showing skin lesions. Dermal papules on this pony were confined to an area of approximately 1 sq. ft., and the disease ran the shortest course of any of the affected animals, *i.e.*, ten days.

CLINICAL PICTURE

Investigation of the feeding program failed to offer a solution. The disease did

The author is on the laboratory staff of the Los Angeles County Live Stock Department, Los Angeles, Calif.

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not respond to various local or internal medications. We first observed the cases on May 25.

The initial lesions in the skin were numerous and usually about the size of a "BB" shot. Although most cases showed these small primary swellings, several animals had early lesions as large as quarters. Swellings were concentric, firm, and neither vesicular nor pustular in nature. Within seven days, the papular lesions became encrusted or covered with scabs, which later dropped off and left circumscribed areas, desquamated and hairless. During the course of the disease, the stages of lesions overlapped in individual animals; new papules would make their appearance as some areas were healing.

The skin disorder caused no pruritus, irritation, or noticeable discomfort. Feed and water consumption were normal, and the general condition and activity of these horses were surprisingly good. The coats, other than the areas affected, remained smooth and bright.

Temperature readings were normal in all affected animals which were examined in all stages of the disease, as far as had been determined. No temperatures were taken before the appearance of lesions.

The course of this disease, as we have seen it, varies from ten days to six weeks, with an average of three and one-half weeks.

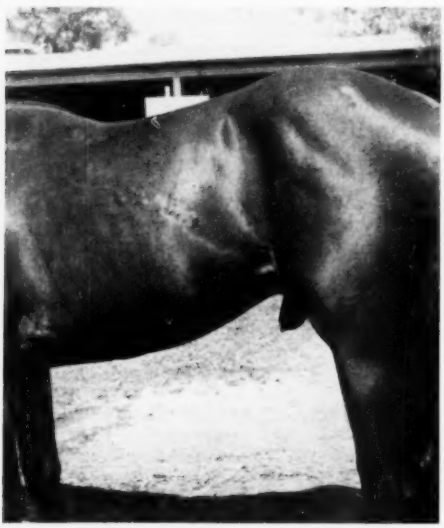

Fig. 2—Papular dermatitis in a Thoroughbred, showing primary stage but larger lesions. Lesions up to the size of a quarter.

MATERIALS AND METHODS

We have not attempted to approach the problem as a strictly research project. Our efforts have been directed more toward making a diagnosis. The procedures used in arriving at a diagnosis were generally "eliminative." In some instances, both tests and materials were limited, and thus invite criticism by numerical errors.

Twenty-three skin scrapings were taken during a period of four weeks. These specimens were examined microscopically for ectoparasites and fungi. Samples were treated with 5 per cent, 10 per cent, and 20 per cent sodium hydroxide solution in both cool and hot digestion.

Skin specimens were also examined culturally with emphasis on cultivation of fungus. Routinely, we used Sabourard dextrose mold agar.

Skin scrapings, including hair, were observed under ultraviolet light in tests for fluorescence. All results were negative for ectoparasites, fungi, or microlarvae.

Seven fecal examinations were not significant.

Six blood films, stained by Wright's method, were negative for blood parasites.

Egg Cultures.—On June 28, fresh, scabby skin scrapings were taken from 3 Thoroughbreds which showed typical lesions. These samples were immediately placed in normal saline solution and returned to the laboratory. Here the macerated scabs were ground in a sterile mortar and the resulting normal saline suspension filtered through a Seitz EK No. 3. Portions of the filtrate were inoculated onto blood agar plates (rabbit blood). These plates were incubated aërobically and anaërobically as a check for bacterial sterility.

Sterile filtrate in 0.1-cc. portions was then injected into four chicken eggs containing 11-day-old embryos. The site of inoculation chosen was the chorioallantoic membrane. For these inoculations, 6 control embryos were held. Eggs were subsequently incubated and candled twice daily. On the fourth day of incubation, 1 inoculated embryo was found dead. The other 3 injected embryos were living but weak, whereas the 6 controls were apparently developing normally. Allantoic fluid and chorioallantoic membrane were harvested from this first dead embryo, a portion of which material was checked culturally for bacterial contamination. The remainder was held under refrigeration for inoculation studies. Seven days following primary egg inoculations, the remaining 3 embryos were dead. Plaques were noted near the site of inoculation in these embryos. The controls were alive at this time and hatched normally.

Animal Inoculations. — In these limited

studies, 5 mice, 3 guinea pigs, and 2 rabbits were inoculated intradermally and subcutaneously with allantoic fluid. Mice received 0.1 cc. and rabbits and guinea pigs, 0.3 cc. These small animals were not affected.

Four mixed grade horses were obtained for experimental inoculation and were held on a premise adjoining the laboratory.

Two mares, apparently in normal health, were selected for study. One was 13 and the other 19 years old. The 19-year-old mare received 0.5 cc. of allantoic fluid, injected intramuscularly and subcutaneously at two sites on the left side of the neck. The other mare received dermal scarification and an intramuscular inoculation of 0.2 cc. of a suspension of chorioallantoic membrane. Again, the sites chosen were on the left side of the neck and shoulder.

Temperature observations were made twice a day and remained in a normal range up to five and one-half days. At this time, the temperature of the 19-year-old mare rose to 105.6 F., while the other mare gave a reading of 102.6 F. The following morning, the 19-year-old mare's temperature dropped to 102.2 F. and the other was up to 103.2 F. The peak temperature, 104 F., was reached in the younger mare six days after inoculation.

No dyspnea or other untoward symptoms were observed in either of these animals prior to, or during, the febrile stage.

Twenty-four hours following the climax of temperature in the oldest mare, approximately, 20 papules appeared over the posterior portion of the shoulder and back on the left side, and a few papules on the right side. No lesions were noted directly at the sites of inoculation.

The 13-year-old mare exhibited a similar picture, primary lesions appearing shortly after the body temperature had returned to a near normal range. The period during which lesions were visible in both of these experimental horses was short, *i.e.*, eight and ten days, respectively.

The animals were slaughtered ten days after inoculation. No gross pathologic changes were apparent. A number of tissues were taken for histopathologic studies. Our studies were not complete on the micropathology at the time this report was written.

To repeat the above trials, 2 grade mares, 6 and 3 years old, were selected. Our route of inoculation was varied in these animals, dermal scarification being the only method selected. Several areas of skin (about 2 sq. in.) were shaved and mechanically cleaned on each mare; one site was on the left side of the neck, the other was behind the left shoulder. Sterile steel wool was used to introduce 1 cc. of the original lot of allantoic fluid into the skin of each mare. Scarification was very superficial.

Temperatures were taken and blood was drawn before inoculation. A much earlier temperature rise occurred in these animals as

Fig. 3—Papular dermatitis of the horse. Slightly beyond tertiary stage (about two weeks from first noticeable symptoms). Scabs have fallen off, showing areas of alopecia.

compared with the first 2 horses. The first skin papules appeared on the 6-year-old mare two and one-half days following dermal scarification; nodules were scattered over the neck, shoulder, and trunk. None developed directly on the site of scarification.

The first evidence of skin lesions in the 3-year-old mare occurred a few hours after their first appearance in the 6-year-old.

All 4 experimental horses manifested a relatively mild form of skin disease as compared to some of the clinical cases previously examined. The extent of the lesions in these trials was, in comparison, approximately one third those of the clinical cases. This relationship is based on the approximate number of papules and areas of skin affected.

Further Egg Cultures.—Skin scrapings were taken from all experimental horses and blood was drawn from 2 mares at the height of the febrile periods. These specimens were filtered through Seitz EK No. 3 and then egg cultured in the manner previously described. Of 30 eggs used in recovery studies, 12 were held as controls. Three eggs for each specimen group were used. Inoculated embryos died within five to nine days. Plaques were noted in the majority of embryos which survived six days.

Serial Egg Passages.—Two series of 5 embryo passages were run. The material in the first group was derived from the original skin scrapings of clinical cases in the Thoroughbreds. Three, 11-day embryos were used in each passage. These inoculations killed all embryos through the fifth passage. Embryos, in groups of 3, died with less than seven hours' variation—at 96, 48, 30, 27, and 26 hours in the first, second, third, fourth, and fifth passages, respectively.

The second series was carried from our first dead embryo used in recovery studies. All embryos died and, again, were rather uniform, in so far as time of death was concerned. Embryos were found dead at 72, 36, 28, and 26 hours in respective transfers.

All egg cultures were checked for bacterial contamination and found to be sterile.

DISCUSSION

A review of the available literature has not offered a description which would resemble the clinical pictures or etiologic agent as we have seen them in these investigations. There are a number of approaches to this disease problem worthy of investigation: trials with other animal species, serologic comparisons with known viruses, immunity studies, and others. Since the Los Angeles County Livestock Department is essentially a diagnostic and disease control organization, we do not plan to carry out any comprehensive research program under our present policies.

SUMMARY

1) A mild form of skin disease affecting Thoroughbreds is described.

2) This disease appears to be highly contagious and infective.

3) Numerous laboratory examinations were undertaken in an effort to recover a causative factor.

4) A virus was isolated by filtration and egg embryo cultures.

5) The virus was inoculated into experimental horses and the disease reproduced, establishing the pathogenicity of the virus under study.

6) Through further egg cultures, the virus has been recovered.

The Effect of Various Compounds upon Horse Strongyle Larvae in Feces

NORMAN D. LEVINE, Ph.D.

Urbana, Illinois

NEMATODE PARASITES of animals are controlled at present either by treatment of the host or by following rules of sanitation designed to prevent the host from becoming infested. A great amount of research has been done on chemical treatment of nematode infestations, and a great deal of information has been accumulated. Relatively little attention has been given, however, to the effect of chemicals on nematode eggs or larvae. A discussion of the problems involved is given by Cameron.[2]

LITERATURE

Fumigation of soil against nematode parasites of plants is standard practice. For this purpose, chloropicrin, carbon disulfide, DD (a mixture of 1, 3-dichloropropylene and 1, 2-dichloropropane), ethylene chloride, methyl bromide, etc. have been used.[3, 4, 5, 10] Fumigants have been tested against the eggs and larvae of animal parasites. Lucker and Schaffer[16] found that carbon tetrachloride, ethylene dichloride, and a mixture of dichloropentanes killed horse strongyle eggs and larvae when mixed with feces. Parnell[24, 25] reported that 0.048 per cent chloropicrin, 0.25 per cent p-dichlorobenzene or o-dichlorobenzene, 0.37 per cent naphthalene, 0.54 per cent dichloropentanes, 4.7 per cent ethylene, 5.5 per cent chloroform, or 8.3 per cent carbon tetrachloride killed horse strongyle larvae in feces. Shumakovich[20, 21] found that 0.1 per cent chloropicrin killed Strongylus larvae in sealed vessels but not in open containers. Andrews, Taylor, and Swanson[1] reported that soil fumigation with methyl bromide under a gas-tight cover, at the rate of 1 lb. per 64 sq. ft., killed pig Ascaris, Strongyloides, Oesophagostomum, and Stephanurus eggs. Swanson and Taylor[34] found that methyl bromide applied in the same way killed all larvae of bovine nematodes to a depth of 12 inches in the soil. Cram and Hicks[6] stated that the fumigation of *Ascaris lumbricoides* eggs in sewage sludge and sand with methyl bromide killed the undeveloped eggs but had little effect on partially or completely developed eggs.

Salt and lye have been used with some success against hookworm larvae. Stoll[33] found that lime mixed with night soil in concentrations from 1:10 to 1:500 killed human hookworm larvae. Oldt[18] stated that human hookworm larvae in night soil were dead after two or more weeks' storage with 25 per cent ammonium sulfate, stone lime, Chili nitrate, or gypsum. Fischer[8] reported that 10 to 20 per cent NaCl solution killed *Ancylostoma duodenale* eggs in a few minutes, and that sodium chloride brine killed hookworm larvae in the soil of mine latrines. Underwood[36] found that *Ancylostoma caninum* larvae were killed in outdoor, bare, dirt pens by saturated NaCl applied at the rate of 0.107 gal. per square foot. McCulloch[17] stated that dog hookworm larvae could be eliminated from kennels by the use of 0.1 gal. of 2 per cent lye solution per square foot. Raevskaja[27] killed dog hookworm eggs on the ground by copious irrigation with 5 per cent phenol.

Several investigators have studied the effect of chemical fertilizers on nematode larvae. The most effective is apparently calcium cyanamide. Rossi[28] found that it killed *A. caninum* larvae on bare runways, and de Giorgi[9] found that a 0.2 per cent solution killed hookworm larvae in Petri dish cultures. Stevenel and Berny[32] stated that 0.3 per cent calcium cyanamide killed hookworm larvae before they hatched in fecal cultures. Parnell[22] reported that a 2 per cent concentration in fecal cultures killed horse strongyle larvae. Leiper[14] found that 0.25 per cent calcium cyanamide killed most horse strongyle larvae in a day in fecal cultures. He found that it killed 59 per cent of the larvae when applied to artificially infested turf at the rate of 500 lb. per acre in an indoor experiment, and that it killed 50 per cent of the larvae in an outside experiment when applied at the rate of 700 lb. per acre. Other, less effective fertilizers, such as kainite (potassium chloride), lime, rock phosphate, and superphosphate have been studied by Lieke[15] and Parnell.[19, 23] Other references are given by Parnell.[25]

Lapage[12] found that various sulfur compounds had little lethal effect on Haemonchus, Ostertagia, and Trichostrongylus larvae. Parnell[20, 21, 22, 24, 25, 26] studied the effect of a large number of substances on horse strongyle larvae in fecal cultures. Those not already men-

From the Department of Veterinary Pathology and Hygiene, College of Veterinary Medicine, and Agricultural Experiment Station, University of Illinois, Urbana.

The technical assistance of Mrs. Betty Maunder is acknowledged.

tioned, which killed in concentrations of less than 1 per cent, were urea (0.80%), aniline (0.19%), calcium cyanide (0.19%), pyridine (0.25%), sodium fluoride (0.67%), sodium silicofluoride (0.67%), and potassium xanthogenate (0.62%). According to Cameron,[2] iodine compounds were found lethal for horse strongyle larvae, 0.01 per cent methyl iodide killing them in feces. Shorb and Habermann[29] found that 2 per cent phenothiazine mixed with feces prevented the development of *Haemonchus contortus* larvae. Threlkeld and Johnson[35] reported that 0.1 per cent phenothiazine prevented the development of *Stephanurus dentatus* larvae in water. Laurans[13] found that copper sulfate had some effect on trichostrongyle larvae from sheep.

In recent years, many new insecticides, miticides, and other compounds have been developed. It was considered worthwhile to determine the effect of some of them upon nematode larvae.

Materials and Methods

A modification of the technique described by Parnell[20] was used in this investigation. Feces were obtained from 6 horses owned by the University of Illinois. The horses were infested predominantly with small strongyles. Of 1,900 sheathed larvae examined at different times, 98.2 per cent were small strongyles with eight intestinal cells (probably Cyathostoma spp.), 1.2 per cent were *Strongylus vulgaris*, and 0.6 per cent were *Strongylus edentatus*. No other types of larvae were seen. No difference in results was observed between the different species of larvae.

Fresh fecal samples were brought to the laboratory, mixed thoroughly, and 36-Gm. aliquots were weighed out. The compounds to be tested were made up in 10 times the desired final concentrations. Those which were sufficiently soluble in water were dissolved in it. Other compounds were mixed with talcum powder to provide bulk. To each fecal sample was added 4 cc. of the liquid or 4 Gm. of the powder under test. The sample was mixed thoroughly and then placed on a square piece of cheesecloth. The square was folded into a bag which was tied with a long string and suspended in a quart fruit jar with the string hanging over the edge of the jar. The jar cover was put on tightly enough to keep the bag suspended, but not so tightly as to prevent air from entering the jar. At least two untreated control samples were set up for each series.

Egg counts were made of the fecal samples by a modification of the Gordon and Whitlock[11] technique on the day that they were set up. The egg counts varied between horses and from day to day for the same horse. In the samples studied, they range from 800 to 7,600 per gram of feces, with a mean of 3,730.

The jars containing the samples were placed in a dark cupboard at room temperature for two weeks. They were then removed, the bags were taken out, and the larvae were collected by the Baermann technique. Each bag was placed in a large funnel, to which was attached a piece of rubber tubing provided with a pinch clamp. The quart jar was rinsed several times with small quantities of warm water, and the rinsings were poured into the funnel. Additional warm water was added to partially cover the bag of feces. The preparation was allowed to stand two hours. The water was then drained into a flask, and a second portion of warm water was poured into the funnel. This was allowed to stand one hour and then was drained into the flask. The volume of the water was measured, and the larvae in it counted.

Larva counts were made of 0.5-cc. samples under the low (x 50) power of the microscope. In collecting samples for counting, the flask was shaken thoroughly, and a sample was withdrawn immediately with a 1-cc. pipette. If the flask were allowed to stand for even a little while, the larvae would begin to settle out. Further, to avoid this source of error in the counts, the lower tip of the pipette was always inserted to the same depth, halfway between the top and bottom of the water. The counts were repeated on fresh samples, until the last count was within 10 per cent of the average of all counts made. Usually, this did not require that more than three or four samples be counted. Since the larvae were alive and moving while being counted, some were undoubtedly missed and others were counted twice. Since the direction of motion was random, the two errors probably cancelled each other.

No claim is made that all the larvae which hatched were recovered by this technique. Indeed, Parnell[21] has shown that changing the water daily for several days did not get them all. For practical purposes, however, the percentage of recovery was considered sufficient. Furthermore, since all samples were handled in the same way, the results can be compared safely with each other. In the control cultures, larvae were recovered from an average of 55.4 per cent of the eggs present in the feces. The range was from 1 per cent to 242 per cent (in the last case, a low egg count was recorded).

After the larval counts were made, the total number of larvae in each specimen was calculated, and the relation of the number of larvae recovered from the treated sample to that recovered from the control sample was determined. To do this, the number of larvae recovered from each treated sample was ex-

TABLE I—Percentages of Horse Strongyle Larvae Surviving Two Weeks' Exposure to Various Compounds in Feces*

Compounds	Concentration of compound in feces							
	10%	5%	1%	0.5%	0.1%	0.01%	0.001%	Other
Inorganic compounds								
Ammonium hydroxide			48		104			
Ammonium chloride			11		53			
Ammonium thiocyanate	0		22					
			0		31	169	133	
Borax	0		0					
Boric acid**			0		53			
Cupric chloride		0		54				
Hydrochloric acid			26		86	50		
Lead arsenate	0		267		182			
Mercuric chloride					0	3		
					0	2		
			0		0	33	17	
			0		0	28	26	
			0		1	16	100	
			0		1	12	74	
Nitric acid			0		124			
Phosphoric acid			0		91			
Potassium bichromate			0		68			
Potassium iodide			0		0			
			0		0	0	152	
			0		0	2	30	
			0		0	1	59	
			0		0	3	119	
Potassium permanganate			70		65			
Silver nitrate			0		78			
Sodium arsenite			0		0	70		
			0		0	0		
			0		0	1		
			0		0	0	83	
			0		0	20	77	
			0		0	2	122	
			1		0	2	82	
Sodium bicarbonate**	0		118					
Sodium carbonate**	0		74					
Sodium chlorate		30		137				
Sodium fluoride**			0		16			
Sodium hydroxide			31		111			
Sodium hypochlorite				71				0.05 %—98 0.005%—95
Sodium iodide			0		0	1		
			0		0	0	91	
			0		0	7	14	
Sodium nitrite			0		40			
Sulfur**	60		73					
Sulfuric acid			2		91	79		
Talcum powder	69 114							
Organic compounds								
Acetic acid			0.3		85			
Amidol			93		135			
ANTU**			62		93			
Arsanilic acid					120			0.3 %— 0
Benzil**			11		38			
Carbarsone**			49		135			
Chloral hydrate			0		119			
Chloramine T			22		205			
Crystal violet					47	211		
Dibutylphthalate	4		43					
	94		114					
Dibutylphthalate plus 0.05% soap			112		96	49		
Diphenylamine	0		22					
Diphenylamine**	0		10					
DDT	58		96					
Ethyl alcohol	0		0					
Formalin			0		12	39		
Gammexane			19		86	92	48	
Hexamethylenamine			64		85			
Hexylresorcinol			0.5		21			
Iodoform**			0		0			
			0		0	31	52	
			0		0	40	88	
			0		0	69	88	
			0		0	63	66	
Kojic acid**			0		6			
Kojic acid**			19		86			
			26		89			
			17		15			
Lethane B-72			0		102			
Nicotine sulfate			0		0			
			0		0	1		
			0		0	0		
			0		0	1	1	
			0		0	1	6	

*Calculated as percentages of numbers of larvae recovered from controls.
**Talcum powder used to dilute the compound in mixing it with the feces.

TABLE I (continued)—Percentages of Horse Strongyle Larvae Surviving Two Weeks' Exposure to Various Compounds in Feces*

Compounds	Concentration of compound in feces							
	10%	5%	1%	0.5%	0.1%	0.01%	0.001%	Other
Orthophenylphenate**			7		26			
Orthophenylphenol plus 1% soap and 0.3% isopropyl alcohol			0		2			
Oxalic acid**			24		138			
Paraphenylene diamine**			0		11			
Penicillin								200 U./Gm.—139
								1000 U./Gm.—116
								1000 U./Gm.—113
								100 U./Gm.—104
								100 U./Gm.—114
Pentachlorophenol**			0		79			
Phenol			0		6	90	87	
			0		4	55	57	
			0		0	118		
Phenol plus 0.5% soap			0		10	35		
			0		16	96		
Phenol plus 0.5% triton X-45			0		0			
			0		9			
Phenol plus 0.5% tergitol penetrant 7			0		6			
			0		4			
Phenothiazine**			0		18			
Phenothiazine plus 1% triton X-45			8		15			
Picric acid (sat. aq. solution)	25		33					
Pyrogallic acid			26		90			
Resorcinol			0		6			
Salicylic acid**			0		32			
Soap				116				
Sulfanilamide**			33		64			
Sulfaguanidine**			66		86			
Tartar emetic**			33		55			
Tergitol penetrant 7			28		92			
Tetraethylpyrophosphate			0		146			
Triton X-45			86		122			
Tyrothricin								0.25 mg./Gm.—71
								0.025 mg./Gm.—90
2-4 D			22	20	65			
Rutgers 612	0		0					
			12		59	26	26	

*Calculated as percentages of numbers of larvae recovered from controls.
**Talcum powder used to dilute the compound in mixing it with the feces.

pressed as a percentage of the number of larvae recovered from its control. These figures are the only ones shown in table 1. To conserve space, the actual larval counts are not given.

RESULTS

The effects of the compounds studied are shown in table 1. In interpreting the figures, a word of caution is advisable. In the controls, a wide variation was noted in the number of larvae recovered from different samples, even though they were prepared and handled in the same way. This same variability has been observed by others.[20] The responsible factors are not known. The fecal cultures in which the larvae develop are complex mixtures of many species of bacteria, molds, yeasts, and Protozoa, of the products of their metabolism, and of materials which have passed through the horse. Little is known of what takes place in such mixtures. Some small, initial difference either in the organisms or organic matter present, or in the environmental conditions, might influence the final population profile markedly. Hence, even apparently large differences in numbers of nematode larvae recovered are not considered necessarily significant.

The following compounds failed to kill all larvae when present in 10 per cent concentration in the feces: sulfur, talcum powder, dibutylphthalate, DDT, and saturated aqueous picric acid solution. Sodium chlorate failed to kill all larvae when present in 5 per cent concentration in the feces.

The following compounds killed all larvae in 10 per cent concentration, but failed to do so in 1 per cent concentration in the feces: ammonium thiocyanate, sodium bicarbonate, sodium carbonate, diphenylamine, and Rutgers 612 (2-ethyl-hexanediol 1, 3).

The following compounds failed to kill all larvae in 1 per cent concentration in the feces, the highest concentration tested: ammonium hydroxide, ammonium chloride, hydrochloric acid, lead arsenate, potassium permanganate, sodium hydroxide, sulfuric acid, acetic acid, amidol, ANTU, benzil, carbarsone, chloramine T, gammexane (the *gamma* isomer of benzene hexachloride), hexamethylenamine, hexylresorcinol, kojic

acid, orthophenylphenate, oxalic acid, phenothiazine, pyrogallic acid, sulfanilamide, sulfaguanidine, tartar emetic, tergitol penetrant 7, triton X-45, and 2-4 D.

The following compounds failed to kill all larvae when present in 0.5 per cent concentration in the feces (no higher concentrations were tested): cupric chloride, sodium hypochlorite, and soap.

The following compounds killed all larvae in 1 per cent concentration, but failed to do so in 0.1 per cent concentration in the feces: boric acid, nitric acid, phosphoric acid, potassium bichromate, silver nitrate, sodium fluoride, sodium nitrate, arsanilic acid, chloral hydrate, formalin, lethane B-72, orthophenylphenol, paraphenylene diamine, pentachlorophenol, phenol, phenol plus 0.5 per cent soap, tergitol penetrant 7 or triton X-45, resorcinol, salicylic acid, and tetraethylpyrophosphate.

The following compounds killed all larvae when present in 0.1 per cent concentration in feces; they failed to kill all larvae in 0.01 per cent concentration: mercuric chloride (in 4 out of 6 trials), potassium iodide, sodium iodide, sodium arsenite, iodoform, and nicotine sulfate.

In addition to the above, 1 per cent borax and 1 per cent ethyl alcohol killed all larvae; while 0.1 per cent crystal violet, 2,000 units per gram of penicillin, and 0.25 mg. per gram of tyrothricin failed to do so.

DISCUSSION

When the compounds under study were mixed with the feces, only the egg stage of the strongyles was present. When the feces were next examined two weeks later, only larvae were searched for. All those seen were third stage. If no larvae were found, it was thus impossible to know whether the compound had acted upon the strongyle eggs, upon the first, second, or third larval stages, or upon all four. Because of its sheath, the third stage larva is usually considered more resistant than the first two. Whether this was the case was not determined in the present investigation. Even if one assumed it, however, he still would not know whether the egg or the first two larval stages were affected. Further investigation would be necessary to determine this point. Since the present study was a preliminary survey, such an investigation was not felt necessary.

It might be remarked further that, in those tests in which no larvae were recovered, it is not certain that they were actually killed, although it is assumed that they were. It is at least possible that development was simply inhibited, and that, if the compound had been removed, development would have proceeded normally.

One of the purposes of the present study was to learn whether some of the newer insecticides might be effective against nematodes. Four were tested. Lethane B-72 ($\beta\beta$-dithiocyano diethyl ether) and tetraethylpyrophosphate were the most effective, 1 per cent concentrations killing all larvae in the feces, but 0.1 per cent failing to do so. One per cent benzene hexachloride failed to kill all larvae, and even 10 per cent DDT was ineffective.

The miticides studied were relatively ineffective. Ten per cent dibutylphthalate and 1 per cent Rutgers 612 failed to kill all larvae.

The rodenticide, ANTU, and the herbicide, 2-4 D, were ineffective in 1 per cent concentration. Another herbicide, sodium chlorate, was ineffective in 5 per cent concentration. The fungicide, pentachlorophenol, failed to kill all larvae in 0.1 per cent concentration, although 1 per cent did so. The antibiotics studied, penicillin and tyrothricin, were ineffective in the concentrations used.

The relative inefficiency of the chlorine compounds and potassium permanganate was not unexpected in the presence of so much organic matter. Two other standard bactericidal agents, formalin and phenol, were also relatively ineffective, 1 per cent of both killing all larvae, but not 0.1 per cent. Hexylresorcinol was even less lethal, a few larvae remaining alive in a 1 per cent concentration. On the other hand, mercuric chloride was among the most effective compounds tested, 0.1 per cent killing all larvae in feces in four out of six tests. In the other two tests, larvae from only 1 per cent of the eggs were recovered.

One per cent sulfanilamide or sulfaguanidine had no appreciable effect on the larvae. Except for hexylresorcinol, mentioned above, the surface active compounds studied showed no anthelmintic activity. One per cent tergitol penetrant 7, 1 per cent triton X-45, and 0.5 per cent soap were ineffective against the strongyle larvae. An attempt to increase the larvicidal activity of phenol by mixing it with 0.5 per cent of each of these compounds was unsuccessful.

Neither hydrochloric, sulfuric, nor acetic acids killed all larvae when present in 1 per cent concentration, but boric, nitric, and phosphoric acids did so. The last three failed to kill in 0.1 per cent concentration, however. One per cent ammonium hydroxide and sodium hydroxide were not lethal, nor were 1 per cent ammonium chloride, ammonium thiocyanate, sodium bicarbonate, or sodium carbonate. One-tenth per cent po-

tassium bichromate, silver nitrate, and sodium nitrite failed to kill all larvae.

Of the four arsenic compounds studied, lead arsenate and carbarsone were least effective, neither killing all larvae in 1 per cent concentration. Arsanilic acid was more effective, killing in 0.3 per cent, but not 0.1 per cent concentration. Sodium arsenite was one of the most effective compounds in the whole series, killing all larvae in 0.1 per cent concentration in all the seven tests made, killing all larvae in 0.01 per cent concentration in two tests, and all but 1 to 2 per cent of the larvae in 0.01 per cent concentration in three tests.

The effect of known nemacides on the strongyle larvae is particularly interesting. Diphenylamine, hexylresorcinol, and phenothiazine failed to kill all larvae in 1 per cent concentration in the feces, while 0.1 per cent sodium fluoride failed to do so. Nicotine sulfate, on the other hand, killed all larvae in 0.1 per cent concentration in five tests. In 0.01 per cent concentration, it killed all larvae in one test, and all but 1 per cent in the other three tests which were made. This finding with nicotine sulfate does not agree with that of Parnell,[25] who reported that 2.9 per cent of the compound was required to kill horse strongyle larvae in feces. All three iodine compounds studied were found highly lethal. One-tenth per cent potassium iodide killed all larvae in all five tests, and 0.01 per cent allowed less than 3 per cent to develop in four tests. One-tenth per cent sodium iodide killed all larvae in three tests, while 0.01 per cent allowed 0.1 and 7 per cent, respectively, to develop. One-tenth per cent iodoform killed all larvae in five tests, but 0.01 per cent of this compound was ineffective.

Before this study was begun, it was thought that perhaps the technique could be used as a cheap and easy method of evaluating and screening potential anthelmintics. The results do not justify such a conclusion. Phenothiazine, for instance, is probably the most effective drug known for horse strongyles. A single dose of 15 Gm. will often kill all strongyles present in the large intestine of a horse. Yet, when 1 per cent phenothiazine was mixed directly with feces, it failed to kill all larvae in one out of two tests. Comparable results were obtained by Shorb and Habermann,[29] who found that it was necessary to mix 2 per cent phenothiazine with sheep feces to prevent the development of *H. contortus* larvae.

SUMMARY

The effect upon the development of horse strongyle larvae in feces of 70 compounds, including various insecticides, miticides, bactericides, fungicides, anthelmintics, antibiotics, and herbicides was studied. The least effective compounds encountered were DDT, dibutylphthalate, sulfur, and talcum powder, which, when mixed with feces in a concentration of 10 per cent, failed to kill all larvae in two weeks. The most effective compounds encountered were mercuric chloride, sodium arsenite, nicotine sulfate, potassium iodide, sodium iodide, and iodoform, 0.1 per cent of which, when mixed with feces, killed all larvae.

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Ulcerative Dermatosis of Sheep

E. A. TUNNICLIFF, D.V.M., M.S.

Bozeman, Montana

THE TERM "ulcerative dermatosis of sheep" is being proposed to cover the group of skin diseases which have been described as lip and leg ulceration, posthitis, balanoposthitis, and ulcerative vulvitis.

REVIEW OF LITERATURE

W. Williams,[1] in 1894, described, under the term orf, or carbuncle, of the sheep in England, a disease occurring more particularly in young sheep, but occasionally in old ones. It is characterized by lameness, inflammation of the coronet or the space between the claws, later developing into angry-looking ulcers which may attain a large size, fill up with granulations, or bleed readily. Similar ulcers may appear on the face and head. They are not fatal, but, when well established, are troublesome and slow to heal. He also used the term "Crusta Labialis" for the affection when the lesions are more marked on the face, although the eruption is seen on the coronets and pasterns as well as the lips and nostrils.

In Great Britain, the venereal form was first described in 1903 by Flook,[2] who relates the presence of extensive eruptions about the mouth and nose and a discharge from the sheath in buck lambs. The affected bucks were placed with a small flock of old ewes, and one week later, 9 of these ewes showed swelling of the vulva, with raw, ulcerating sores on the skin and mucous lining of the lips of the vulva.

McFadyean,[3] in 1903, also of Great Britain, observed the same disease affecting the vulva of ewes with the production of swelling, ulceration, and discharge. He reproduced the disease by collecting the discharge on cotton, which was placed into the sheath of a wether. On the third day, a small sore, covered by a brownish scab, appeared on the skin near the opening. A number of small ulcers formed, covered by brownish crusts. The author did not succeed in isolating any organism which he believed caused the disease, but considered the disease worthy of careful observation, and was convinced that newly purchased bucks might well be examined for this affection before being used in breeding.

G. H. Williams,[4] in 1904, reported on two more outbreaks of this disease in England, affecting the genital organs of bucks and ewes and similar to those recorded by the 2 preceding writers.

Knowles,[5] in 1907, described very fully a disease, occurring among the sheep of southeastern Montana, which affected the lips and legs of the animals. He succeeded in transferring the disease from infected to healthy sheep by a series of inoculation experiments as early as 1904. He was the first writer to apply the appropriate name, "infectious lip and leg ulceration," to this disease. His observations and descriptions are so accurate and the information so timely that a part of his writing is quoted:

". . . . with lip and leg ulceration, as it occurs in Montana, it is with us a distinctly fall and winter disease. I have observed it as early as the latter part of August and continuing until the following April, but the time of greatest prevalence is mid-winter . . . when our range forage is succulent and green, the disease does not appear. We do not observe it in low lying or swampy localities; in fact, its first appearance in Montana was among two bands of sheep . . . at an elevation of at least 3,500 ft. All outbreaks in Montana have occurred in our northern and eastern tier of counties, among mountains or high rolling prairies; in no case at an altitude of less than 2,500 ft. In any of these localities, however, may be found swampy areas extremely limited in dimension; usually in the vicinity of a stream, lake, or so-called pot hole. During the prevalence of the disease in Montana, all such places are frozen over; and at this time, usually, the sheep depend upon snow for moisture, at intervals subsisting upon range forage and snow for two or three months at a stretch without access to water.

"Sheep of all ages are susceptible and I have observed no apparent difference in severity attributable to age; but among sheep on short range and indifferent water, necessarily poorly nourished, the disease is most severe and fatalities relatively greater. Perhaps 60 per cent is the largest number of infections observed by me in any one band of sheep, the average being from 15 to 20 per cent. Mortality is normal where sheep are on good range,

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In this investigation, the author made all of his determinations in ml.; 1 ml. is equal to 1.000027 cc.

well nourished, and the ulcers are properly treated.

"If a leg be the point of infection, the sheep shows lameness early and, as the ulcer progresses, will lag behind the band; an examination at this time will show a well progressed, necrophorus, vascular ulcer, usually in the vicinity of the coronet; or between the coronet and knee, with plainly apparent swelling and acute inflammation of contiguous tissue. If necrotic area of skin at point of infection has not been disturbed, it will be found covering the ulcer, lid like, lightly attached by threads of connective tissue, and about the loosened edges, a thick, creamy pus exudes.

Fig. 1—Lip and leg ulceration field case, illustrating typical circumscribed lesion.

"If promptly treated, progress of the disease is cut short and the ulcer rapidly heals; if neglected, destruction of tissue rapidly progresses until the ulcer may cover an area of an inch or two and, if over the coronary articulation, not infrequently penetrates the joint, in some instances observed complete disarticulation of one or both coronary joints has occurred.

"If the lip be the seat of infection, it will be observed that the sheep is not feeding, or is feeding indifferently and with apparent effort. Examination will show swelling, radiating from point of infection, hard and tense, a necrotic skin area dependent upon the extent of primary infection; and in not longer than sixty-four hours, a well defined, necrotic, vascular ulcer, identical pathologically with that of the leg. The lips and legs (usually the anterior) are the ordinary points of infection, although in a few cases observed one or both eyes have been infected, ultimately resulting in total destruction of the eye or eyes."

Mohler[6] published an excellent article in 1910 on lip and leg ulceration of sheep. It is still a classic in spite of the fact that the necrophorus organism was considered the causative factor. Tidswell,[7] 1910 to 1911, wrote of the necrosis bacillus with special attention given to "balanitis" (sheath disease) of sheep in New South Wales. Glover,[8] 1923, described and discussed lip and leg ulceration and "anovulvitis." Welch,[9] 1926, published on lip and leg ulceration and venereal disease of bucks and wethers in connection with the sore mouth-necrophorus complex. Belschner,[10] 1930, wrote that the condition, pizzle disease, was not contagious. Puller,[11] 1931, suggested a filterable virus as the cause of posthitis. Bosworth and Glover,[12] 1931, described the occurrence of the venereal type of disease in Great Britain. By transmission tests, they showed the difference between it and contagious pustular dermatitis. From Idaho,[13] 1935, foul sheath in rams was described. Steyn,[14] 1936, wrote of pisgoed or pisgras in South Africa and transmitted the

Fig. 2—Lip lesion, ewe E-10, experimentally transmitted from an experimental posthitis case.

disease by pus, but not by pure cultures from the lesions. The description corresponds to the lesions of posthitis in this country. Filmer,[15] 1938, described the treatment of pizzle rot. Beveridge,[16] 1941, did not believe the American type of posthitis the same as Australian pizzle rot. Tunnicliff and Matisheck,[17] 1941, reported the etiologic agent of posthitis to be a filterable virus. Roberts and Bolton,[18] 1945, published on a venereal disease of sheep that seems to be the same as our posthitis or balano-

Fig. 3—Interdigital lesion of contagious ecthyma, field case.

posthitis. However, the authors did not think it the same as the Australian pizzle rot.

DESCRIPTION OF DISEASE

Characterization.—Ulcerative dermatosis of sheep is an infectious condition characterized by epidermal and subcutaneous tissue destruction, resulting in the appearance of raw, granulating ulcers affecting the skin of the lips, legs, feet, and genital organs of both male and female sheep.

Location of Lesions.—These ulcerative processes are usually located between the borders of the upper lip and the nasal orifice, the pastern above the coronet dorsal to, or involving, the interdigital pouch, the interdigital space itself, the lips of the vulva, the prepuce at the orifice, and the glans penis. Other lightly wooled areas of the body may develop ulcerative lesions.

Pathology.—The process is primarily one of tissue destruction, consisting of an ulcer containing a leucocytic mass covered by a slightly raised, hard, brownish scab. The lesions are circumscribed, varying from 20 to 45 mm. in diameter, with a depth of 3 to 10 mm. (fig. 1 and 5). The base and walls of the ulcer are composed of highly vascular tissue, spotted with grey and red necrotic areas. The leucocytic mass is odorless with a thick, creamy consistency.

Lip and leg ulceration lesions are fundamentally the same as those given in the preceding description (fig. 1). However, those that occur above or surrounding the interdigital pouch may not be circumscribed, but of a spreading, irregular nature (fig. 3). Lesions on the interdigital space may not show ulceration, but a moist, grey coagulation necrosis confined to the tender skin between the toes.

Posthitis, balanoposthitis, and ulcerative vulvitis lesions do not vary from the general tissue destruction type of process (fig. 2 and 4 to 9). In our experience, by the time the condition is discovered, the circumscribed ulcers have coalesced to form a continuous lesion, partially or entirely surrounding the preputial orifice. The process is accompanied by considerable swelling and edema due to the highly vascular nature of the tissue (fig. 4). We have never encountered lesions on the inner surface of the prepuce. Extreme cases result in phimosis. On the glans penis, the ulcers develop moist, glistening scabs that are unlike the dark, dry ones on the prepuce that have been exposed to the drying effect of the air (fig. 6). Ulcerative vulvitis is the same type of lesion as posthitis, starting at the dorsal tip of the vulva and progressing upward on the lips of the vulva. Some edema usually accompanies the formation of the ulcer and there is a tendency for it to be less circumscribed (fig. 7 to 9).

Etiology.—The causative agent of posthitis, balanoposthitis, and ulcerative vulvitis is a filterable virus. Five infective, bacteria-free filtrates have been prepared by passage through two Berkefeld N candles, one Berkefeld V, a 7-lb. Mandler candle, and a 3½ per cent collodion membrane. The gross pathology of the resulting experimental lesions was typical in every way of the field cases of posthitis and the experimental lesions produced by nonfiltered tissue suspensions.

Without adequate proof, the necrophorus organism was considered the etiologic agent of the entire posthitis lip and leg complex for many years. In the very beginning of our posthitis investigations, stained slides from diseased tissues showed them to be remarkably free from the necrophorus organisms, in particular, and the pus-producing bacteria, in general. Subsequent observa-

tions have verified these findings. A few gram-positive diplococci and some small gram-negative rods are found. However, the fresh, young lesions usually are quite free from bacteria and the necrophorus organism is almost never present.

EXPERIMENTAL

The experimental data to follow are presented to show the intertransmissibility and

Fig. 4—Posthitis, ram 631, experimentally infected from posthitis lesion on ram 630.

similarity between the two conditions that have been hitherto classed as separate disease entities, *i.e.*, the posthitis, balanoposthitis, ulcerative vulvitis complex, and lip and leg ulceration.

Evidence is also offered in substantiation of the original announcement of the filterability of the etiologic agent of balanoposthitis by Tunnicliff and Matisheck.[17]

Posthitis, Balanoposthitis, and Ulcerative Vulvitis.—In November, 1940, 6 ewes and rams (1757 to 1761), affected with ulcerative vulvitis, posthitis, and balanoposthitis, were obtained from a sheep outfit near Livingston, Mont. Ulcers on the vulva, prepuce, glans penis, and, in one instance, the lip were extensive, severe, and typical of such infections. Ram lamb 1758, having an ulcer on the glans penis, and ewe lamb 1761, with an ulcer on the vulva, were used in the following transmission tests.

A suspension of the material from the ulcers on the glans penis (1758) was rubbed into the scarified skin on the prepuce and glans penis of ram 513 (fig. 6), the skin on the lips of the vulva of ewe E-67 (fig. 8), and the skin on the upper lip of ewe E-104. In the same manner and with a suspension of the ulcerated material from the vulva of ewe lamb 1761, ram 10 and ewe E-145 (fig. 9) were inoculated on the scarified skin of the prepuce and vulva, respectively. All 4 of the sheep developed ulcers typical of posthitis and ulcerative vulvitis. The ulcers on the lips were exactly like the other lesions.

By serial inoculation, the virus suspension used to inoculate ram 10 and ewe E-145 was carried through 11 transfers on the skin of the prepuce of rams before being tested on the lips of sheep. The prepuce lesion suspension was then inoculated into the scarified skin of the lip of ewe E-10 (fig. 2), resulting in a typical ulcerative process. The material from this lesion later reproduced a characteristic preputial lesion on ram V-1115.

In December, 1942, material from ulcerative vulvitis lesions was obtained from some ewes near Cut Bank, Mont. The tissue suspension of a vulva lesion was rubbed into the scarified skin of the prepuce of ram V-1037 and the skin of the lips of the vulva of ewe N-67. Following the development of typical ulcerative processes on both sheep, the necrotic, ulcerous

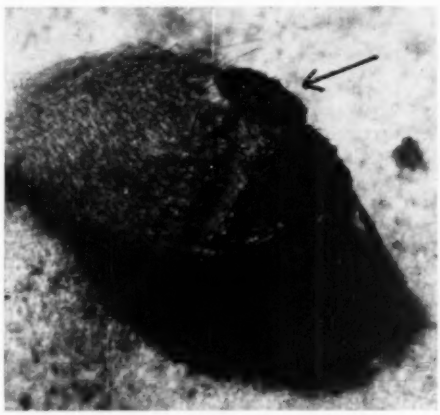

Fig. 5—Posthitis, ram 629, experimentally transmitted from posthitis lesion on ram 631. Arrow points to circumscribed freshly developed ulcer.

tissue obtained from the vulva lesion of ewe N-67 was transferred to, and reproduced posthitis lesions on, the skin of the prepuce of ram 653. A suspension of this ulcerated tissue was transferred to the scarified skin of the vulva and lips of ewe N-120. Severe, typical ulcerous lesions developed at the site of both inoculations.

Material was again obtained in March, 1943, from the same band of sheep near Cut Bank.

Tissue specimens were obtained from ulcerative vulvitis and balanoposthitis lesions. They were pooled and the resulting suspension inoculated into the scarified skin of the lip and vulva of ewe N-79 and the skin of the prepuce and lip of ram 659. All four sites developed severe and typical ulcerative processes. Diseased tissue from the skin of the vulva and

Fig. 6—Balanitis, ram 513, experimentally transmitted from field balanitis case 1758.

prepuce of ewe N-79 and ram 659 was collected, pooled, and inoculated into the scarified skin of the lips of the vulva and upper lip below the nostril of ewe N-16. Both the vulva and upper lip inoculations produced characteristic ulcers. Material from the upper lip ulcer reproduced typical ulcers on the skin of the vulva and upper lip of ewe N-16.

One of our sheepmen from Rosebud, Mont., presented ewes and rams that were affected with posthitis, balanoposthitis, and ulcerative vulvitis in January, 1943. They had typical and severe ulcers on the skin of the prepuce, glans penis, vulva, and upper lip. An emulsion from the ulcer on the skin of the prepuce of 1 ram reproduced typical lesions on the scarified skin of the upper lip and prepuce of ram 649. A suspension from the lesion on the upper lip of 1 of the affected ewes produced lesions on the scarified skin of the prepuce and lip of ram 650. An emulsion prepared from the ulcer on the lip of the vulva produced typical lesions on the scarified skin of the upper lip and lip of the vulva of ewe V-1115. The scarified skin of the upper lip and vulva of ewe N-75 was inoculated with a suspension of the prepuce lesion of ram 650. Characteristic lesions developed on both inoculation sites.

Lip and Leg Ulceration.—In March, 1940, a field investigation was made of lip and leg ulceration in a band of ewes near Ryegate, Mont. It was possible to obtain material from cases in the early stages and transmission tests were started immediately. In the affected band of 1,650 ewes, most of the lesions were confined to the lips, although there were a few leg lesions. All of the new cases developed lesions on the upper lip, some extending to the nostril. The experimental infections involved the same area on the lip, with very little disposition toward a spread to other parts of the body. The process consisted of a raised, hard-crusted scab, varying in size from 1 to 15 mm. in diameter. Upon removal, a crater of vascular granulation tissue, covered by creamy, odorless pus, was revealed. Caseation was not evident.

A normal saline suspension was prepared from the newly developed lip ulcers for transmission tests. Between March and July, a series of five such tests was carried out, in each case using the lip as the inoculation site. Transmission trials to the male or female genital organs were not attempted, for, at that time, the similarity of posthitis and lip and leg ulceration was not recognized. The resulting lip lesions were typical of the naturally occurring lip and leg ulceration.

Fig. 7—Ulcerative vulvitis, ewe 3000R, experimentally transmitted by copulation with a ram having a natural case of balanoposthitis.

At the time of the investigation of an outbreak of lip and leg ulceration near Martinsdale, Mont., February, 1943, 250 affected ewes had been cut out of some 750. Almost all of the affected ewes had foot or leg lesions, the majority of these being ulcers above the coronary band and either anterior or lateral. There were a few affected feet showing interdigital ulceration. Some ewes had lip ulcers located on the upper lip just below the nasal orifice. A characteristic lip lesion from the upper lip of 1 of the affected ewes was collected for inoculation trials, as was one of the typical ulcers from an area dorsal to the interdigital space.

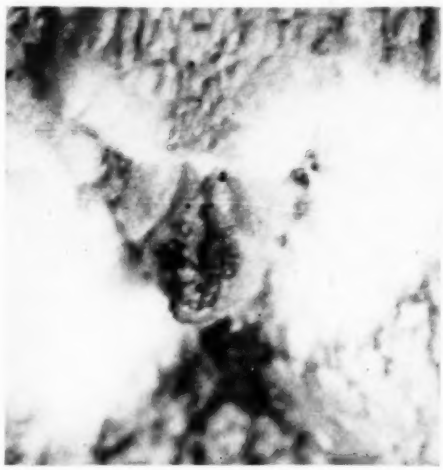

Fig. 8—Ulcerative vulvitis, ewe E-67, experimentally transmitted from field case of balanoposthitis on ram 1758.

The lip lesion suspension was inoculated into the scarified skin of the upper lip, the lips of the vulva, and the skin of the lower pastern of ewe N-28. Active ulceration resulted in all three inoculation sites. From the pastern lesion, a tissue suspension was prepared that was inoculated into the scarified skin of the upper lip and the pastern and prepuce of ram 657. The same inoculum was used for exposure tests with ewe N-37 when the scarified skin of the lips of the vulva, the upper lip, and the pastern were inoculated. All six inoculation sites on the ram and ewe developed active, typical ulcerative processes.

In November, 1944, an investigation was made of lip and leg ulceration in ewes near Willow Creek, Mont. There were about 50 cases among some 5,000 ewes. The majority of the lesions were on the feet, between the hoof and the fetlock joint, with very few on the face.

A second field investigation was made a week later, at which time it was found that about 30 more sheep showed lesions, principally on the legs. Two rams were found with sheath lesions. However, within a period of seventeen days after the beginning of the breeding season, at least 27 rams showed ulcerative lesions of the prepuce. No vulva lesions were observed within the first three weeks of the outbreak and, according to the owner's statement, none appeared later. Actually, this may not have been the case, for, in view of the posthitis lesions present in the rams, it does not seem possible that the ewes would have escaped infection.

One ewe with lesions on the upper lip and both forefeet and ram 2549 showing preputial lesions were brought to the laboratory for study. The lesions on the foreleg and lip of the ewe were the characteristic ulcerative processes typical of lip and leg ulceration. A tissue suspension from the lip lesion produced typical ulcerative processes on the prepuce of ram S-2002 and the upper lip of ewe P-17. From the prepuce lesion of ram S-2002, the infection was transmitted to the lips of ewes P-83 and N-32. The prepuce lesion (2549) was the typical degenerative process characteristic of lip and leg ulceration. An emulsion from this lesion again reproduced a typical lesion on the upper lip of ewe A-1228.

Fig. 9—Ulcerative vulvitis, ewe E-145, experimentally transmitted from field case of ulcerative vulvitis on ewe 1761.

Filtrates.—Infectious, bacteria-free filtrates were prepared from posthitis and vulvitis lesions (1757 to 1761), obtained from the outbreak of posthitis, balanoposthitis, and ulcerative vulvitis in sheep near Livingston, Mont.

Suspensions of the prepuce (1758) and vulva lesions (1761) were pooled to be inoculated onto the prepuces of 2 rams and the vulvas of 2 ewes. These diseased tissues were then collected, pooled, and inoculated into the scarified skin of the prepuce of a ram. From this case of posthitis, transfers were made to the prepuce of a second ram and to a third one (1054-E). In each case, the process was a typical, severe ulcer. From the prepuce of the last ram (1054-E) of this series, characteristic, raw ulcerative tissue was collected from which filtrate (1803) was prepared as follows: An emulsion was made from the prepuce lesion by trituration with alundum in a solution consisting of equal parts of 5 per cent horse serum water and beef broth, pH 8.2. After centrifugation, the supernatant fluid passed through a Mandler candle. However, the sterility tests revealed bacterial contamination.

The prepuce lesions of rams E-63 and 179-E, inoculated with a tissue suspension (1803), were allowed to develop for a week, at which time the granulating ulcers had become severe and extensive. The scab and granulation tissue

Fig. 10—Contagious ecthyma in lamb H-113, seven days after experimental inoculation. Note the proliferation of tissue.

(1805) of the ulcers was collected and triturated in a solution of equal parts of 5 per cent horse serum in water and beef broth, pH 7.7, from which the supernatant fluid was separated by centrifugation. It was then passed through glass wool before being filtered through a 3 per cent collodion membrane. The filtrate proved infectious, as shown by the posthitis lesion on inoculated ram 606. However, subcultures of the filtrate showed it to be contaminated with bacteria.

The infection was passed from ram 606 to ram D-2056 by means of a suspension of the posthitis lesion, producing, on the seventh day, an active, progressive, and severe ulceration from which tissue suspension 1825 originated.

The tissue, debris, blood, and edematous fluid collected from the ulcer curettage constituted the suspension that was triturated with alundum in a solution of equal parts of a 1 per cent horse serum-water broth mixture. This was centrifuged and the clear supernatant fluid, pH 7.05, filtered through Berkefeld N and W, and a 7-lb. Mandler candle. Serum agar slants, seeded with 0.5 to 1.0 cc. of the filtrates, remained sterile throughout the six days' incubation at 37 C. Each of these three bacteria-free filtrates was infective. Characteristic, severe cases of posthitis in rams 617, 620, 614, and 615 resulted, with the exception of ram 620, in which case, the lesions were less severe, though typical. Rams 617 and 620 received the Berkefeld W filtrate, and 614 and 615, the Mandler and Berkefeld N filtrates, respectively. The infection was further transmitted by means of a tissue suspension of the posthitic ulcer on ram 615 to ram 613, with the typical posthitis resulting.

Another infective, bacteria-free filtrate

Fig. 11—Contagious ecthyma in lamb H-113, eighteen days after inoculation. Note extensive tissue proliferation and heavy scab formation.

(1870) was obtained April 30, 1941, from a composite of the prepuce lesions from rams E-63, 179-E, and 607. The lesions were small, mild, and retrogressive at the time of their collection. Trituration in distilled water and alundum resulted in a heavy suspension infectious for ram 623. The suspension was diluted in 7.5 parts of beef broth, pH 8.2, centrifuged, and passed through a 3½ per cent

collodion membrane. Filtrate-inoculated serum-agar slants were sterile eight days later. Prepuce inoculation of ram 626 resulted in mild lesions that were active and typical. The infection was then transmitted to the scarified skin of the sheath of ram 632.

An infectious, bacteria-free filtrate was prepared from a prepuce lesion obtained from the Rosebud, Mont., outbreak. Ram 650 was inoculated on the scarified skin of the prepuce with a suspension of the lip lesion from one of the naturally affected ewes. The prepuce developed an extensive, severe ulcerative lesion which was harvested on the eleventh day and prepared for filtration as follows: Four grams of soft, ulcerated tissue were triturated in 4 cc. of 5 per cent sheep serum water, alternately quick frozen with a mixture of CO_2 and 95 per cent ethyl alcohol and thawed seven times at nine- to thirteen-minute intervals, until the last time, which was seventeen minutes. The frozen suspension was then thawed, 0.6 cc. placed in 2-cc. tubes, quick frozen and desiccated at 35-45 μ for 125 minutes on a lyophylizing apparatus. The resulting residue, from 2.4 cc. of the original suspension, which had been a 1:1 suspension, was diluted with distilled water to a 19 per cent suspension that was then clarified in an angle head centrifuge at 4,200 r.p.m. for one hundred minutes before passage through a ¾ in. length Berkefeld N candle. The yield amounted to 7 to 8 cc., which was cultured on serum-agar slants and determined free of bacteria after ten days' incubation at 35 C. The scarified skin of the prepuce of ram 654 was then swabbed with the filtrate. Seven days later, an active, typical, ulcerous posthitis process had developed, which was later transmitted, by an emulsion of the lesion, to the prepuce of ram 656.

DIFFERENTIAL DIAGNOSIS

With the ordinary cases, there need be no difficulty in arriving at a diagnosis of ulcerative dermatosis. However, it is possible to confuse it with the lip and foot lesions of contagious ecthyma, the interdigital space lesions of foot rot, and the noninfectious, pizzle rot type of posthitis. Fundamentally, the basic difference is that they are entirely pathologic processes. Ulcerative dermatosis causes tissue destruction (fig. 1 and 5 to 8); whereas, sore mouth results in tissue proliferation (fig. 10 and 11). Foot rot is a definite necrotic process.

The primary lesion on the skin of the interdigital space in both foot rot and ulcerative dermatosis infections is that of a moist coagulation necrosis, which, if entirely confined to the interdigital space and in the absence of any other lesions, will be impossible to differentiate. If the lesion involves the bulb of the heel undermining the sole of the foot and extending into the vascular laminae of the horny wall, then one need have no fear in making the diagnosis of foot rot.[19] Another point of differentiation is the characteristic foul odor of the young coagulation-necrosis lesion of foot rot on the interdigital space. This odor is not present in ulcerative dermatosis and, furthermore, foot rot is always limited to the feet.

In our experience, the virus of ulcerative dermatosis attacks only the epidermal tissue, seldom, if ever, extending to the mucous membrane. The virus of sore mouth has a predisposition for the lips at the junction of the mucous membrane and the epidermis (fig. 10 and 11), extending to the face (fig. 12) and interdigital space beneath the interdigital pouch (fig. 3).

Fig. 12—Contagious ecthyma in lamb C-84, natural infection, illustrates necrosis and heavy scab formation on skin of lips and face.

The noninfectious type of posthitis has been observed to occur only on the skin surrounding the preputial orifice, the glans penis remaining unaffected. This is logical since it is assumed that the resulting lesion is caused by irritation from dusty ground or filth with which the end of the sheath may come in contact. When the lesion is confined to the end of the prepuce, there is no sure method whereby one may differentiate between infectious and noninfectious posthitis; with the possible exception that noninfectious posthitis is benign, nontransmissible, and does respond more readily to treatment than the infectious type.

Bacteriologically, the lesions of ulcerative dermatosis are quite free from the necrophorus organism and all ordinary pus-producing bacteria. Foot rot and sore mouth

lesions both contain quite a collection of miscellaneous bacteria, and the necrophorus organism is usually present. It is certainly a secondary invader in the case of sore mouth, and, as yet, its rôle is not entirely determined in the case of foot rot.[20,21]

Bosworth and Glover,[12] in 1931, presented proof of the etiologic difference between sore mouth and venereal infection by means of cross immunity experiments.

An immunity experiment was set up at our laboratory in 1940 for the purpose of determining if the primary cause of lip and leg ulceration might be the same as the virus of contagious ecthyma. Three yearling ewes, which had been vaccinated with ecthyma vaccine and later proved immune, were inoculated with the lip and leg material. At the same time, 3 other nonvaccinated yearlings were inoculated. In this experiment, the vaccinated sheep developed lesions comparable to those produced in the nonvaccinated animals, proving that lip and leg ulceration was not produced by the same virus as contagious ecthyma.

DISCUSSION

The lip lesion, from a natural case of lip and leg ulceration in a ewe, was reproduced with typical ulcer formation on the lip and prepuce of a ram and the vulva, lip, and pastern of a ewe. The experimentally inoculated prepuce lesion was again reproduced on the lip of a ewe in the form of a typical ulcer. A tissue suspension from the experimental pastern lesion again reproduced typical ulcers on the lip, pastern, and vulva of a ewe and the lip, pastern, and prepuce of a ram. A naturally occurring prepuce lesion of a ram, also having lip and pastern lesions, was reproduced on the lip of a ewe. An ulcer on the pastern of a ewe affected with lip and leg ulceration was experimentally produced on the lip, vulva, and pastern of another ewe and the lip, prepuce, and pastern of a ram.

Typical posthitis lesions were reproduced on the glans penis, prepuce, and lips of rams, and the vulva and lips of ewes from cases of posthitis, balanitis, and ulcerative vulvitis. Naturally occurring ulcers on the lip of a ewe produced typical lesions on the lip and prepuce of a ram. Inoculation with the prepuce ulcer suspension resulted in lip and vulva lesions on a ewe. Characteristic lip and vulva lesions were produced by the material from an experimental vulvitis case that had been infected from a ram with a natural balanitis infection. The infection from an experimental case of vulvitis (inoculated from a naturally infected one) was transmitted to the prepuce of a ram and onto the lips and vulva of a ewe.

The foregoing résumé of intertransmissibility has shown that the identical type of ulcerative lesion was produced experimentally from both the lip and leg ulceration and the posthitis types of the disease. Additional proof was obtained from several field investigations that confirmed the experimental results. By way of illustration, typical lip ulcers were found among the sheep in a band that was suffering from a characteristic posthitis outbreak in which the majority of lesions were located on the prepuce, glans penis, or vulva. Likewise, an outbreak of lip and leg ulceration occurred among ewes just before breeding season, the lesions being confined to the lips and legs. However, within seventeen days after the rams were placed with the ewes, 27 of them showed ulceration of the prepuce, indistinguishable from the venereal type of posthitis.

Considering the intertransmissibility of the infections and the exact duplication of lesions, the logical conclusion is that the two diseases, hitherto considered separate entities, are in reality different manifestations of the same disease. The term "ulcerative dermatosis of sheep" is proposed to include all of these conditions.

Infective, bacteria-free filtrates, prepared from typical ulcers characteristic of posthitis and ulcerative vulvitis, reproduced ulcers on the lips and legs that were indistinguishable from those that were produced on the vulvas and prepuces by the same filtrates.

Definite proof of the presence, in lip and leg ulceration lesions, of a virus which will produce both types of disease is lacking because the filtrates that did produce characteristic lesions were later found to have been contaminated. This phase of the work is incomplete due to lack of suitable field material.

SUMMARY

1) The posthitis, balanitis, ulcerative vulvitis complex, and lip and leg ulceration conditions are different manifestations of the same disease for which the name "ulcerative dermatosis of sheep" is proposed.

2) The etiologic agent of the posthitis complex is a filterable virus.

3) Conclusive proof is lacking on the etiology of lip and leg ulceration, yet, in view of the following information, it is quite probable that the cause of the disease will be proved to be the same virus:

a) The intertransmissibility of identical lesions from experimental and field cases.

b) Infective, bacteria-free filtrates from posthitis and vulvitis cases have

produced lesions identical with those found in lip and leg ulceration infections.

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DISCUSSION

CHAIRMAN MURPHY: Thank you, Dr. Tunnicliff.

Are there any questions from the floor regarding this presentation?

DR. L. R. VAWTER (Reno, Nevada): Have you made any attempts at prophylactic immunization, Dr. Tunnicliff? We have a great many inquiries on that point.

DR. TUNNICLIFF: Our work is not a bit good on that. The few immunity tests we have conducted have indicated no immunity produced by this lip and leg posthitis virus. I can cite several field cases where terrific outbreaks occurred. I followed those sheep through and tagged them, and in about six months—in fact, around five months—that same thing broke out again in the same band, and some of the same sheep that already had had one dose of it came back with the second case of posthitis. At the present time, it looks as though there is no immunity produced by it.

Experimental Avitaminosis A in Sheep

D. F. EVELETH, Ph.D., D.V.M., D. W. BOLIN, M.S., and A. I. GOLDSBY, B.A.

Fargo, North Dakota

THE SHEEP husbandry practices in the Northwest are such that at times sheep are forced to exist for long periods of time on rations low in carotene. During the spring, summer, autumn, and even into the winter, the sheep are pastured, but the growing season is short and after severe freezing in October or November, there is no green grass until the following May or June. Thus, the sheep are grazed on stubble fields or cut-over hay fields, allowed to eat straw, or fed hay for a period often exceeding seven months. The type of hay fed is often very low in carotene. The other feeds, except when yellow corn is fed, are practically devoid of carotene.

As an aid in diagnosis of nutritional diseases, an attempt was made to produce the symptom-lesion complex resulting from feeding sheep a ration furnishing all of the nutrient requirements except carotene.

The descriptions of the symptoms and lesions of avitaminosis A of sheep are incomplete and to a certain extent contradictory. Vawter[1] described a disease syndrome resulting from insanitation and the feeding of a ration consisting entirely of sheaf oats. Prostration and coma were the symptoms noted.

Hart,[2] in his review on vitamin A, does not stress the symptoms other than night blindness in sheep. Schmidt[3] states that the symptoms of vitamin A deficiency in sheep and goats differ somewhat from those in cattle. He lists loss of appetite, loss of weight, and night blindness as common symptoms. In other cases, there are nasal discharges, anemia, opacity of the cornea, and convulsions but no edema. The survival period of mature sheep on the carotene-low ration varies from 273 to 779 days. Sudden deaths and urinary calculi are frequently the termination of the vitamin A deficiency syndrome. In 1944, Foss and Eveleth[4] reported on field cases of pregnant ewes in which ketosis was a common finding, with the ewes responding to vitamin A therapy. There were, however, certain symptoms in these cases that were different from those reported by Schmidt. There were no urinary calculi and in many instances there were ascites and subcutaneous edema.

EXPERIMENTAL

Most of the data presented here were obtained on grade ewes which started the experiment as yearlings. The ewes were all from one flock that had been wintered on a grain and alfalfa hay ration. The ewes were selected, identified, and placed on a ration of straw, soybean meal, dried beet pulp, oats, and dehydrated potatoes. Later, the potatoes were omitted as it was impossible to obtain a constant supply. Tap water was supplied *ad libitum*, as were minerals. Sodium chloride mixed with sodium iodide and cobalt carbonate, bonemeal, and ground limestone were fed in separate containers.

TABLE 1—The Serum Vitamin A Content Following Single Doses of Carotene as Compared with Nonsupplemented Ewes.

Sheep (No.)	I.U. per 100 cc. serum			
	Oct. 31	Nov. 1	Nov. 22	Jan. 23
233	42	24	24	56
234	42	26	22	48
236	36	28	38	60
239	40	36	38	74
240	20	12	18	28
242	52	48	38	56
243	30	24	18	48
246	36	12	38	42
235	16 (30,000)*	23 (49,950)	42 (49,950)	60
238	65 (60,000)	80 (49,950)	46 (49,950)	108
241	32 (90,000)	74 (49,950)	80 (49,950)	54
245	24	22 (49,950)	60 (49,950)	47
247	24 (3,330)	24	40	47

*I.U. carotene given.

The sheep were weighed at biweekly intervals and periodic blood samples were taken for analyses. When a definite avitaminosis A was known to be present, various amounts of carotene were given by capsule to some of the sheep.

Other data, such as cerebrospinal pressure, wool clip, carcass grade, night blindness tests, and later, the ability to conceive were obtained on the surviving animals.

A group of lambs, taken at weaning time, was used in the same type of study, but the lambs became depleted very rapidly and most of them died of intercurrent infections.

The ewes were maintained in dry lots. An anthelmintic was given when the sheep were started on the experiment and they were practically parasite free, as shown at postmortem

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examination. The sheep were all vaccinated against ovine ecthyma.

The chemical methods used are listed in the references.[6-10] The cerebrospinal pressure was determined by a modification of the method described by Sykes and Moore.[11]

The sheep were fed the carotene-low ration for 175 days before any carotene supplements were given. At this time, 6 sheep were selected and given capsules containing carotene in a cereal base.

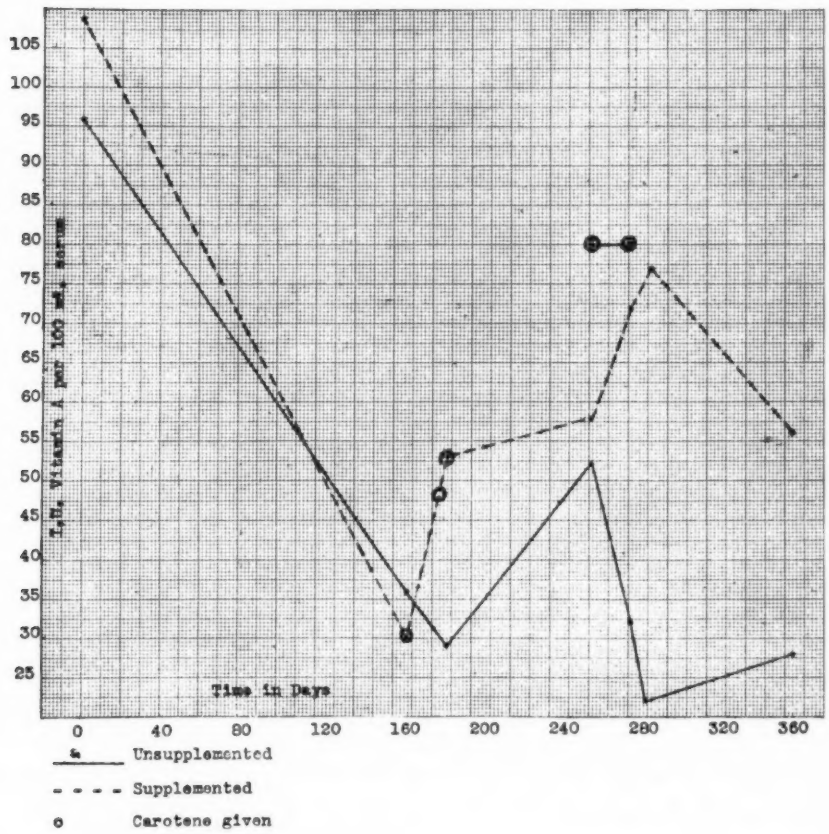
The results of single doses of carotene given at various periods are shown in table 1. The serum vitamin A values of 4 sheep fed 30,000 I.U. per day for twenty days as compared with the serum vitamin A values of the unsupplemented controls are shown in chart 1.

The rapid rate of vitamin A disappearance from the blood and its storage in the liver is shown in table 2. On Nov. 4, 1947, the vitamin A content of the serum in the 2 sheep was nearly identical, while the liver vitamin A of the supplemented ewe was 10 times that of the unsupplemented ewe. In this and other experiments, it was not possible to demonstrate carotene in the blood serum of sheep.

In table 3 (taken from Bolin and Bolin[5]), are shown the effects of feeding thiourea to sheep and then dosing with carotene. These data illustrate the failure to convert carotene to vitamin A in thiourea toxicosis but, in these cases also, it was impossible to demonstrate carotene in the blood serum.

Some data on the vitamin A content of sheep liver are presented in table 4. The livers of newborn lambs contained very little vitamin A but lambs on good pasture had liver vitamin A of over 500 I.U. per gram.

CHART 1—Serum vitamin A content of carotene-supplemented, and nonsupplemented, vitamin A deficient sheep



The data indicate that if the liver vitamin A falls to less than 20 I.U. per gram there is a definite deficiency.

The weights of the control and supplemented sheep are shown in table 5. It is apparent that, under the conditions of this experiment, the addition of carotene had only an insignificant effect on the weight.

Table 6 presents a comparison of the carcass grade and wool clip of the two groups of sheep. While the average grades and pounds of wool clipped for the supplemented group are slightly higher, the differences are not marked.

TABLE 2—Serum and Liver Vitamin A Content Following the Feeding of Carotene to a Vitamin A Deficient Ewe as Compared with the Unsupplemented Control

Oct. 26—				
Serum vitamin A	16	U/100 cc.	*24	U/100 cc.
Nov. 1—				
Serum vitamin A	12	U/100 cc.	128	U/100 cc.
Nov. 4—				
Serum vitamin A	16	U/100 cc.	17	U/100 cc.
Nov. 4—				
Liver vitamin A	1.8	U/Gm.	18.4	U/Gm.

*199,000 I.U. carotene fed.

The cerebrospinal pressure and the serum vitamin A content for 7 sheep are shown in table 7. The normal cerebrospinal pressure is about 65 mm. of cerebrospinal fluid, while it is usually much higher for sheep showing signs of vitamin A deficiency.

TABLE 3—The Effect of Thiourea on the Conversion of Carotene to Vitamin A by Sheep

Sheep (No.)	Vitamin A content of serum I.U./100 cc.				
	Jan. 21	**Feb. 17	Feb. 18	Feb. 19	Feb. 20
233	188	109	103	109	123
3809	95	68	75	103	90
7566	*93	35	36	24	23
7567	*93	35	42	42	35

*Thiourea 6 Gm. per day, Jan. 21 to Feb. 17, 1948.
**All 4 sheep given 3,330 I.U. carotene per pound body weight.

Table 8 presents data obtained on the hemoglobin, total serum protein, serum globulin, and serum albumin. The data do not indicate that under the conditions of these experiments there is any marked change in the concentration of the blood proteins.

On Nov. 6, 1947, the surviving ewes were regrouped as shown in table 9. At this time, ewes 234 and 239 had been on the deficient ration for 546 days. The others had been supplemented as shown in table 1 and chart 1. The ram was put with them on Nov. 26, 1947.

The symptoms of vitamin A deficiency in sheep are difficult to describe. One of the first symptoms noted was a progressive excitability. The sheep were easily startled and difficult to handle. Pica was present as shown by their chewing on the fences. Some sheep died suddenly without showing symptoms. They remained in good flesh. Those that were deficient over a prolonged period of time developed an incoördination with a tendency toward lordosis. The heads were frequently held quite high. Night blindness was one of the first symptoms noted, but corneal opacity was very slow to develop. Opacity of the cornea was noted in only a few of the ewes even in the terminal stages of vitamin A deficiency.

TABLE 4—Vitamin A Content of Sheep and Lamb Liver

Lambs at birth	I.U. per gram	Lambs on pasture	I.U. per gram
1	0.72	901	259.0
2	0.46	1669	237.2
3	0.46	A248	503.6
4	1.36		
5	1.18		

Depleted lambs	I.U. per gram	Sheep on low carotene diet	I.U. per gram
418	17.0	233	1.0
1698	8.2	236	25.9
417	0.72	237	1.8
		240	18.6
		214*	18.4
		245**	7.3

*199,000 I.U. carotene given.
**Given carotene as follows: Nov. 11, 49,950 I.U.; Nov. 21, 49,950 I.U.; from Jan. 25 through Feb. 14, 30,000 I.U. daily; from March 3 to death, 30,000 I.U. daily.

The gross pathology produced by prolonged carotene deficiency is in no way pathognomonic.

TABLE 5—Average Weights in Vitamin A Deficient and Carotene-Supplemented Sheep

Days on deficient ration	Control	Carotene supplemented
0	89	91
175	111	114
186	113	116
196	114	115
261	120	120
281	121	120
298	115	116

A rather typical description of a ewe killed *in extremis* 335 days after starting the carotene-low ration is given below.

Ewe 240 was moribund on April 9, 1947. Live weight 82 lb.; killed by section of jugular veins and carotid arteries; carcass well cov-

TABLE 6—Carcass Grade and Wool Clip of Vitamin A Deficient and Carotene-Supplemented Sheep

	Control	Supplemented
Pounds wool per sheep	11.6	12.4
Grade	4.0	4.8

ered with subcutaneous fat; no signs of subcutaneous edema; hemorrhages in the subcutaneous tissues of the legs; mammary gland typical of virgin ewe; oral cavity, no gross pathology; laryngeal lymph nodes markedly enlarged with a few hemorrhages present; small petechiated hemorrhages on the epiglottis; eyes grossly normal; optic nerve of left eye showed mild constriction in region of optic foramen; nasal passages slightly inflamed; thyroid enlarged and very difficult to cut; pericardium slightly thickened; epicardial and endocardial hemorrhages; lungs congested with several hemorrhages, one of which was 3 cm. wide; spleen of normal size but capsule thickened; rumen contained about a gallon of ingesta; recticulum grossly normal; omasum had small hemorrhages in the folds; abomasum contained small amount of fluid, inflamed in region of pyloric valve; liver was light colored with some hemorrhages, friable; gall bladder normal; ureter of left kidney enlarged about 4 diameters; both kidneys swollen; no urinary calculi; pylorus edematous; hemorrhages in mucosa of duodenum; reproductive system grossly normal; carcass weight 48 lb.; liver vitamin A content 18.6 I.U. per gram.

TABLE 7—Cerebrospinal Fluid Pressure of Vitamin A Deficient and Carotene-Supplemented Sheep

Sheep (No.)		Cerebrospinal fluid (mm.)	Vitamin A I.U./100 cc. serum
239	Vitamin A deficient	152	32
240	Vitamin A deficient	116	30
243	Vitamin A deficient	123	20
246	Vitamin A deficient	70	30
241	30,000 I.U. carotene daily	55	136
245	30,000 I.U. carotene daily	64	112
265	Normal alfalfa hay	65	

DISCUSSION AND SUMMARY

The course of the vitamin A deficiency syndrome in nearly mature ewes is strikingly different from that in lambs. Preliminary experiments have shown that most lambs die in three to four months on a carotene-low ration. Intercurrent infections appear to be very important in causing the death of the lambs. The data obtained in these experiments indicate that, where well-fed yearlings are concerned, the time of depletion is extremely variable and, for approximately a year, there is little influence on weight.

Blood-serum vitamin A increases rather rapidly when deficient animals are given small amounts of carotene and also is rapidly removed for storage in the liver. The normal vitamin A content of the serum appears to be about 100 I.U. per 100 cc. A liver content of less than 20 I.U. appears to be subnormal.

A low serum vitamin A content appears to be associated with an increase in the cerebrospinal fluid pressure.

Vitamin A deficiency does not appear to markedly influence the ability of the sheep to put on fat or to produce wool.

Ewes maintained on low carotene rations have the ability to conceive and, when furnished additional carotene, to carry the lambs to term and to produce milk.

A few tests showed that ewes in advanced stages of vitamin A deficiency may

TABLE 8—Average Blood Protein Fractions in Vitamin A Deficient and Carotene-Supplemented Sheep

Date	Days on deficient ration	Hemoglobin* C	Hemoglobin* S	Total protein* C	Total protein* S	Serum globulin* C	Serum globulin* S	Serum albumin* C	Serum albumin* S	Albumin/globulin ratio C	Albumin/globulin ratio S
May 9	0	10.2	11.3								
June 17	40	10.4	10.8								
Aug. 7	91	11.3	11.4								
Sept. 19	134	11.7	12.2								
Oct. 26	171	12.2	12.5								
Nov. 1	176	11.9	12.3								
Nov. 22	197	12.4	13.3								
Jan. 23	259	12.5	13.3	7.0	7.1						
Feb. 4	271	12.6	13.3	7.5	8.0	3.8	4.7	3.7	3.3	1.0	0.7
Feb. 10	277	12.4	12.5	7.1	7.8	4.9	4.5	3.7	3.5	1.1	0.8
Feb. 17	284	12.6	12.9	7.7	7.8	4.0	4.5	3.2	3.4	0.9	0.8
Feb. 28	295	12.1	12.5	7.0	7.0	3.2	3.8	3.2	3.2	0.8	0.9
Mar. 14	309	11.8	12.8	7.3	7.1	3.8	3.6	3.6	3.5	1.0	1.0
Apr. 4	330	12.0	12.6	6.1	7.7	4.1	3.9	3.7	3.8	0.9	1.0
Apr. 11	337	12.1	12.0	8.0	7.6	4.2	4.0	3.7	3.6	0.9	0.9
May 12	368	11.7	12.4	8.2	7.3						

*Gm. per 100 cc. C = Controls. S = Supplemented.

or may not have a ketonuria. In no case, did the ewes show evidence of subcutaneous edema. As shown in table 1 and chart 1, the nonsupplemented controls showed a marked increase in serum vitamin A on the 198th day. This was due to the animals being accidentally fed some prairie hay. This single supplement of carotene caused a

TABLE 9—Survival and Breeding Record of Carotene-Supplemented, and Nonsupplemented, Vitamin A Deficient Ewes

Sheep (No.)	Carotene I.U. (daily)	Remarks
234	38,000	Died Jan. 10, 1948. Single pregnancy. Severe gastroenteritis.
238	38,000	Single pregnancy. Put on pasture with lamb May 3, 1948.
239	38,000	Single pregnancy. Weak. Lamb died May 3, 1948.
247	0	Died Jan. 12, 1948. Nonpregnant. Pneumonia and abscess in lungs.
241	0	Died March 15, 1948. Twin pregnancy. Fatty liver.

rise in the serum vitamin A that did not return to the previous low for nearly sixty days.

The gross pathology produced by prolonged vitamin A deficiency may be very slight and is in no way constant. Tissues saved from many of these animals may show histologic changes that are not apparent in the gross tissues.

The outstanding symptom of vitamin A deficiency appears to be incoördination and an apparent weakness. The animals have difficulty in breathing after strenuous exercise and may fall when attempting to run. Night blindness appears to be a common symptom but permanent blindness is very slow to develop.

In no case, have urinary calculi been observed in ewes and lambs dying from chronic vitamin A deficiency.

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DISCUSSION

VOICE FROM FLOOR: At what age did you start the lambs on experiment?

DR. EVELETH: At weaning time. Some of those lambs had a little grass, but for the most part the ewes and lambs were on a ration composed of prairie hay, oats, and linseed meal. At 2½ to 3 months of age, the lambs were taken from the ewes and put on a ration of linseed meal, oats, beet pulp, and dried skimmilk.

VOICE FROM FLOOR: How long did it take these lambs to break and show definite symptoms?

DR. EVELETH: Some of the lambs were given supplements of carotene at the start of the experiment. Those not supplemented started to show symptoms of the deficiency within five to six weeks.

DR. J. O. SCHNAUTZ (Oregon): Any disturbances in the pituitary organ?

DR. EVELETH: No gross disturbances. Histologic studies are not completed.

DR. L. R. VAWTER (Nevada): How far have you carried the relationship of vitamin A to fertility?

DR. EVELETH: We had 5 ewes on deficient rations for three months. Four of these conceived; 1 did not. Apparently the prolonged vitamin A deficiency did not interfere with the ability to conceive. It did, however, with the ability to reproduce since in that group of 5 ewes, we got but 1 lamb. Two of the ewes were not supplemented. Of the 3 that were, 1 died; 1 had a lamb and it died; and 1 had a very weak lamb. We saved it by putting the ewe on alfalfa pasture.

DR. SCHNAUTZ: Were any blind at birth?

DR. EVELETH: No. They appeared perfectly normal.

DR. G. H. BARRY (California): Ordinarily, on the range, where there is a little green feed and dry pasture with some brush, would sheep get the proper amount of carotene or vitamin A?

DR. EVELETH: Under most conditions, we would not be likely to see vitamin A deficiency in ewes that had gone through one reasonably good year. From our study, we conclude if the ewe can go through one good grass season, it will have enough liver storage to carry over a considerable time.

MR. MILLER: In our work, some 5-year-old Rambouillet ewes were heavily charged with carotene, then put on this reduced diet, and it was twenty-three months before they showed an appreciable deficiency of vitamin A.

DR. EVELETH: I would think phosphorus and protein deficiency would be more important on extremely dry ranges than an actual deficiency of vitamin A or carotene.

DR. H. H. COLE (California): I am interested in Dr. Eveleth's description of fence chewing. We have observed it in cattle on perfectly good rations. When alfalfa hay and grain are fed in a finely ground state, bone or fence chewing may develop within ten days. In other words, the physical condition is an important factor in whether or not the cows chew fences and two-by-fours. You were feeding straw. I wonder if your animals were eat-

ing it, or if failure to eat the straw would explain the fence chewing?

DR. EVELETH: They would nibble at the straw, but we kept the mixture of soybean meal or linseed meal and grain before them at all times and they did not eat very much straw.

VOICE FROM FLOOR: Did you feed ground grain?

DR. EVELETH: No.

MR. MILLER: I believe that has a bearing on it. We fed cottonseed and mixed the grain with the hulls so the sheep had to eat the hulls with the grain. We did not see a deficiency such as chewing of fences until some prolonged feeding of this ration.

DR. EVELETH: These sheep did not chew fences for nearly a year.

CHAIRMAN MURPHY: Thank you, Dr. Eveleth.

A Toxemic-Uremic Syndrome in Baby Pigs Fed on Dried Skimmilk

W. W. GREEN, Ph.D., H. C. H. KERNKAMP, D.V.M., M.S.,
M. H. ROEPKE, Ph.D., and L. M. WINTERS, Ph.D.

St. Paul, Minnesota

IN CONNECTION WITH studies on the artificial raising of baby pigs, a severe and often fatal metabolic disturbance resulted when certain diets were fed. The disturbance displayed the characteristics of a toxemia that was manifested by nausea, vomiting, diarrhea, dehydration, itching, muscular jerks, stupor, and coma. Associated with these symptoms was a high degree of nitrogen retention in the blood, a uremia. The diets which produced the disturbance consisted chiefly of fat-free milk solids reconstituted with water in various proportions, and the severity and course of the syndrome varied with the levels at which this substance was fed.

The over-all objective of the study concerned itself with the development of feeding or dietary schedules, for baby pigs that were separated from their dams at an early age, which would stimulate or provoke rates of growth and bodily development superior to those that occurred under the natural growing conditions. The dietary schedules selected, which form the basis for this report, failed to meet one of the most important aspects of the objective, namely, that of maintaining, in full measure, a healthy and vigorous pig. Because many of the clinical, chemical, and pathologic findings that occurred in connection with this artificially induced toxemic-like disturbance resembled some of the clinical, chemical, and pathologic findings observed in a naturally occurring toxemic and uremic disease of baby pigs, it seemed desirable to report the results of these studies.

PROCEDURES AND THE BASAL DIET

Baby pigs of inbred lines of stock were used for these experiments. They were separated from their dams when approximately 7 days old, grouped or lotted, placed in wire bottomed cages, and managed as described by Green *et al.*[1] An interval of two and sometimes three days was allowed between the time of weaning and experimental feeding. Pasteurized whole cow's milk (CM) containing 3½ per cent butterfat was supplied during this period. The pigs appeared healthy and vigorous when the experiments began. Blood was collected for chemical analysis (according to the technique of Carle and Dewhirst[2]) just prior to feeding the experimental diets and at weekly intervals thereafter, or more frequently when indicated. The blood urea nitrogen (BUN) was determined by the aëration method,[3] blood sugar according to Nelson,[4] and hemoglobin by the oxyhemoglobin method using the Evelyn photoelectric colorimeter. The BUN and blood sugar are reported as milligrams per 100 cc. of blood and hemoglobin as grams per 100 cc. of blood. Statistical procedures used were those given by Snedecor[5] and statistical significance is indicated by probabilities based on the 5 per cent ($P = < 0.05$) and 1 per cent ($P = < 0.01$) levels. Standard deviations are indicated with some of the means. Although the number of observations for computing some of the means was quite low in some cases and, therefore, not wholly accurate,[6] nevertheless, they are given to indicate the amount of variation.

The basal diet was a fluid mixture of spray process, dry fat-free milk solids (FFS), 15 per cent by weight and tap water, 85 per cent by weight. The powder was thoroughly dispersed by means of a power stirrer. To all rations used in this study, there was added an acidified aqueous solution of mineral salts at the rate of 1 cc. per pound of pig per day. The mineral solution contained 0.1 mg. per cubic centimeter of each: cobalt sulfate, copper sulfate, and manganese sulfate, and 22.5 mg. of ferrous sulfate. Five cubic centimeters of U.S.P. cod liver oil per pig per day was given by floating it on the surface of the fluid diet. The feed was available at all times and a fresh supply was given the pigs each day.

The 15 per cent FFS ration was chosen chiefly because it qualitatively approximated fresh cow's skimmilk and because sow's milk has been reported to contain about this amount of fat-free solids. This diet provoked the toxemic-uremic syndrome in a very high pro-

Paper No. 2438 of the scientific journal series of the Minnesota Agricultural Experiment Station. From the Divisions of Animal Husbandry and Veterinary Medicine, University of Minnesota, University Farm, St. Paul.

Associate professor of animal breeding, Division of Animal Husbandry, University of Minnesota (Green); professor of veterinary medicine, Division of Veterinary Medicine, University of Minnesota (Kernkamp and Roepke); and professor in charge of animal breeding, Division of Animal Husbandry, University of Minnesota (Winters).

Dr. Green, at present, is professor of animal husbandry, Animal Husbandry Department, University of Maryland, College Park.

TABLE I—Survival Rate of Baby Pigs Fed Various Rations

Lot No.	History Ages in days on preliminary ration	No. of pigs	Basic ration	Ages in days while on ration	Days on ration	Mortality due to basic ration no. of pigs / days on ration	Per cent Survival*
7*	3½% CM 7-10	11	15%FFS	10-21	11	1/11	91
7*a	lot 7 split at 21 da.	5	15%FFS	21-56	35	2/14 1/20	40
7*b	lot 7 split at 21 da.	5	3½%CM	21-23	2		100
7*c	5 pigs cont. from 7*b	5	7%CM	23-40	17	2/1 1/2 1/11	20
7*d	1 pig cont. from 7*c	1	15%FFS	40-51	11	1/11	0
9*	3½%CM 6-8 days	4	15%FFS	8-29	21	1/18 1/19	50
9*a	2 pigs cont. from 9*	2	3½%CM	29-56	27		100
12	3½% CM 8-10 days	8	15%FFS	10-20	10	1/8 5/9 2/10	0
12	3½% CM 4-6 days	2	15%FFS	6-14	8	1/3 1/8	0
14	3½% CM 9-14 days	2	15%FFS	14-38	24	1/23 1/24	0
14	3½% CM 8-13 days	2	15%FFS	13-27	14	1/7 1/14	0
14	3½% CM 7-12 days	1	15%FFS	12-14	2	1/2	0
14	3½% CM 6-11 days	2	15%FFS	11-16	5	1/2 1/5	0
20	3½% CM 7-9 days	7	15%FFS	9-16	7	2/3 3/4 1/5 1/7	0
23	3½% CM 7-10 days	10	15%FFS +11%D	10-29	19	2/2 5/4 1/8 1/19	10
23a	1 pig cont. from 23	1	15%FFS+ 8%D+1%Y +1%Cg	29-56	27		100
10	3½% CM 9-11 days	4	15%FFS+ 3%Karo†	11-56	45		100
11	started basic ration	11	3½%CM	8-24	16		100
11a	lot 11 split at 24 da.	6	3½%CM	24-56	32	1/22	84
11b	lot 11 split at 24 da.	5	15%FFS	24-56	32	1/5 1/6 1/7 1/12	20
13	started basic ration	8	3½%CM	7-32	25	1/6	88
13a	7 pigs cont. from 13	7	15%FFS	32-46	14		100
13b	lot 13a split at 46 da.	4	3½%CM	46-56	10		100
13c	lot 13a split at 46 da.	3	15%FFS	46-56	10		100
8*	3½% CM 6-8 days	5	7% CM	8-20	12		100
8*a	Cont. from lot 8*	5	3½%CM	20-29	9		100
8*b	2 pigs cont. from 8*a	2	3½%CM	29-56	27		100
8*c	2 pigs cont. from 8*a	2	15%FFS	29-56	27		100
16	3½% CM 7-12 days	2	10%FFS	12-46	34		100
21	3½% CM 7-10 days	11	10%FFS	10-33	23	1/18	91
21a	lot 21 split at 33 da.	5	10%FFS+ 8%D	33-56	23	1/11 1/15	60
21b	lot 21 split at 33 da.	5	10%FFS+ 1%Y	33-56	23		100
28	started basic ration	10	10%FFS	9-26	17	1/8	90
28a	9 pigs cont. from 28	9	7%FFS	26-56	30		100
24	3½% CM 7-10 days	10	10%FFS+ 8%D+ 1%Y+1%Cg	10-56	46	1/13	90
25	started basic ration	7	10%FFS+ 8%D	7-56	49	3/3 1/4 1/23	28
27	started basic ration	10	10%FFS 8%D+	9-56	47	5/6 1/9 1/13	30
22	3½% CM 6-10 days	11	20%FFS	10-17	7	1/2 5/3 3/4 1/5 1/7	0
26	10%FFS + 8%D 7-9 da.	7	20%FFS+ H_2O ad lib	9-56	47	2/6 1/8 due to 10%FFS+ 8%D	100
5	varied rations 7-11 da.	2	30%FFS	11-13	2	2/2	0
5	3½% CM 13-15 days	3	30% FFS	15-19	4	3/4	0

CM = 3½% butterfat pasteurized cow's milk; FFS = spray process fat-free milk solids; D = dextrin; Y = dried brewer's yeast; Cg = cereal grass.

*Minnesota No. 1 pigs, others Poland China pigs.

†Pigs dying from causes other than those ascribed to the basic ration are listed as survivors.

‡At 11 days, 5 Gm. Y per pig per day and graduated up to 12.5 Gm. Y per pig per day at 44-56 days. At 22 days, 0.12 Gm. of vitamin C per pig per day started.

portion of the pigs that received it. The results were unexpected and an attempt was made to discover some of the reasons for its occurrence.

EXPERIMENTAL RESULTS

The duration of feeding of the different rations, the morbidity, and the mortality of the various lots of pigs are given in table 1. The BUN values are shown in table 2 and in figure 1. Averages only are used in those instances where numbers involved are low, and the average is a reasonably accurate indication of the amounts of BUN. The graphs in figure 1 are based on average values for the days plotted, except for the groups which got 10 per cent FFS plus 8 per cent dextrin (D). In the latter case, the lot means are shown individually because a graph based on averages would mean little.

Ration A.—Five different lots (7, 9, 12, 14, and 20), containing a total of 39 pigs, were started on a ration of 15 per cent FFS. The pigs were 6 to 14 days old when first put on this ration. Two pigs of lot 7 survived on this ration until 56 days old. Two others survived to 56 days (lot 9a) but were changed to 3½ per cent CM at 29 days of age, after the other 2 pigs of lot 9 had died. The 2 that were changed were very sick at the time the switch to 3½ per cent CM was made. Of the 39 pigs, 36 either died or were sacrificed when moribund; and all showed, in varying degrees, one or more of the clinical and pathologic characteristics which were associated with this syndrome. In addition, one group of pigs (lot 19) was given the 15 per cent FFS ration and its members were sacrificed, 1 each day, in order to study the changes which took place. All of these pigs became sick after being on the diet three days, and 1 died on the third day.

In general, the pigs partook of the 15 per cent FFS diet quite readily for the first three or four days. By the fifth and sixth days, many of the pigs, and especially those started on the experimental diet when 6 to 8 days old, manifested, to a greater or lesser degree, clinical evidence of a disturbed metabolism that became more exaggerated with time. The results showed very clearly that the younger the pigs are when started on the diet the shorter is the interval before symptoms are displayed and also that the course of disease is more rapid than in the case of the pigs which are older when first placed on the experimental feed. For example, pigs that were from 6 to 10 days old when started on the 15 per cent FFS diet sickened and died on the average seven and six-tenths days later, whereas, those started when from 11 to 14 days old averaged eleven and three-tenths days. In some cases, the disease was acute and ran its course in three to four days, and in others, it was chronic and extended over ten days. In addition, some differences were noted between pigs of different lines of breeding.

The clinical picture of those that sickened on this diet was quite similar in a majority of the cases. About the first indication that the pigs were not normal was that the coat lost its sheen and the hairs stood more erect and gave the coat a wooly appearance. There was a tendency to rub or scratch the bodies against the sides of the pen and to scratch the medial surface of one hind limb with the hoof of the other. Many showed muscular jerking by extending a hind limb and producing several short kicking movements. The ears drooped and were cold to the touch. Their general attitude manifested a sense of depression and lassitude. Nausea and retching and sometimes vomiting occurred. The bowel evacuations were loose and many developed a diarrhea. The rectal temperature was not elevated. With the progress of the disease, the signs of general physical weakness became more noticeable. There was a tendency to remain recumbent and, in many, coma preceded death. The comatose state often lasted for twenty-four hours and more.

The blood chemistry studies showed that the BUN values rose rapidly and continued to rise as long as the pigs were kept on the 15 per cent FFS diet (fig. 1). The mean BUN value for the group just prior to being placed in the experiment was 10.5. After receiving this diet for five days, the mean value for those living was 23.2. For those living on the eleventh day of the experimental feeding, the mean value was 29.0; for the seventeenth day, 32.0; for the twenty-second day, 41.0; and for the thirty-second day, 43.5. The blood sugar values for the same period did not show marked differences. The mean value at the beginning of the experiment was 108.6; after five days on the experiment, it was 92.0; eleven days, 88.2; seventeen days, 103.9; and 114.0 the twenty-second day. No quantitative or qualitative data were obtained on the urinary secretions. This was unfortunate but, under the conditions of the experiment, satisfactory urine specimens could not be obtained.

The necropsy findings varied somewhat depending upon the duration of the syndrome. The general physical condition of the body or the indication as to the state of nutrition was fair to poor. The hair was dry and coarse and the tail and buttocks usually soiled with fecal material. The kidneys, as a rule, were enlarged, pale, soft, and friable and the capsule stripped off easily. Cross section of the kidney often revealed collections of urates in the renal pelvis and, in many instances, the cortical zone was much lighter than the medullary

zone. The microscopic changes were those which suggested a degenerative process, more especially characterized by granular and hydropic degeneration of the uriniferous tubules. With appropriate staining, globules of fat were found to be accumulated in the epithelial cells at the distal end of a nephron and in some which lined the pelvis. The urinary bladder, except that it sometimes contained urine in which flocculation of urate material was suspended, did not show gross injury. The liver usually was dark, but, in cases of longer standing (ten days or more), it was mottled with yellowish areas. In some instances, it was definitely fatty. Histologically, the liver cells showed degenerative changes similar to those in the

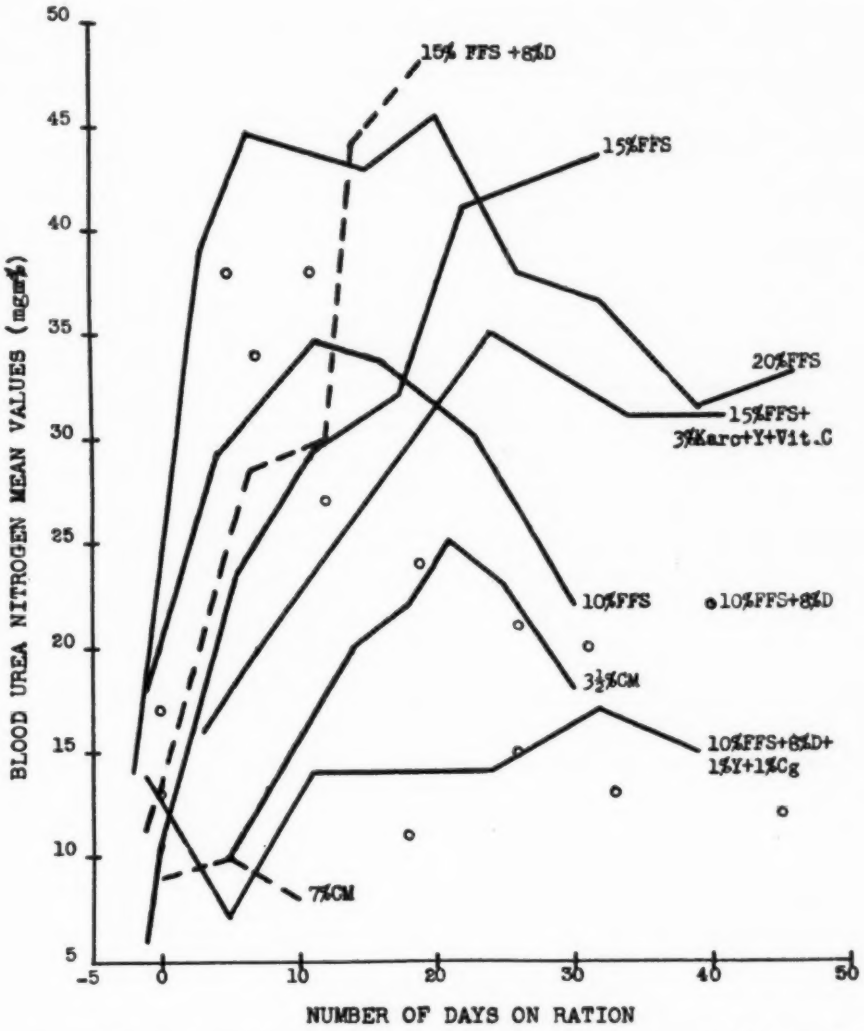

Fig. 1—Blood urea nitrogen values of baby pigs fed diets containing various amounts of fat-free milk solids.

FFS = fat-free milk solids (spray process). D = dextrin. CM = cow's milk (pasteurized). Y = dried brewer's yeast. Cg = cereal grass (dehydrated).

kidney tubules. When fatty metamorphosis was found, the fat did not occur in any special area of a lobule with regularity. The adrenals, in most cases, were enlarged and pale. Greater or lesser amounts of fat were usually found in this organ. The heart was usually enlarged and the musculature pale, soft, and flabby. Upon microscopic examination, it showed signs of degenerative change. The mesentric blood vessels were engorged and the lymph nodes in the great mesentery were swollen and pale. In many of the cases, the walls of the stomach were flaccid and toneless, and very often the longitudinal folds or plica of the mucous membrane lining it were not visible or only faintly discernible. The mucous membrane was pale and it appeared to be very thin. Microscopic sections of the stomach wall showed the crypts to be low and broad with a low grade inflammatory reaction in the lamina propria. The mucous membrane of the small intestine, and especially the duodenal and jejunal portions, was slightly hyperemic. The brain, in most cases, was swollen with evidence of edema when examined microscopically. There were no gross changes in the mouth, esophagus, and the respiratory tract.

In general, the clinical, blood chemistry, and necropsy findings suggested that a metabolic disturbance of the nature of a toxemia was responsible for the syndrome. Associated with this, and which may be a very significant factor in connection with the over-all picture of the disease, was the matter of the high degree of nitrogen retention.

Ration B.—With the view of widening the nutritive ratio of the basal diet for the purpose of noting what effects it might have on the general condition of the pigs and the BUN levels, a mixture containing 15 per cent FFS, 11 per cent D, and 74 per cent tap water by weight was prepared. Dextrin was prepared by Stein Hall and Company and known as W. C. dextrin. It was kept in a satisfactory suspension by first, selecting a product which would stay in suspension quite well and second, by stirring it thoroughly with a power stirrer for twenty minutes.

A group of 10 pigs (lot 23) was used to test this diet. For three days, between the time of weaning and the time they were placed on the test diet, the pigs were fed on CM containing 3½ per cent butterfat. Two of the pigs died the second day after receiving the experimental diet; 5 died or were sacrificed because of their extreme prostrate condition on the fourth day; 1 died on the eighth day, and 1 on the nineteenth day. The remaining pig (B982) was very sick on the nineteenth day, with a BUN of 45.3 and a blood sugar of 105.5. At this time, it was transferred into lot 24 where it remained until discharged as recovered, twenty-seven days later.

Two of the lot died before an examination of their blood was made. By the fourth day of the experimental feeding, 6 of the 8 pigs remaining were very sick. At this time, the BUN of the 6 sick pigs averaged 197.4 and the blood sugar 104.0. The BUN was only slightly elevated for the 2 pigs that were not clinically sick on this date. However, it rose to relatively high levels within the following ten days when 1 came to its death. The physical symptoms and postmorten findings were those we associate with the toxemia-uremia syndrome in the acute form.

It is quite obvious that the addition of D was far from being beneficial; rather, it increased the toxicity of the diet.

Ration C.—Lot 10 (4 pigs) was fed a diet containing 15 per cent FFS, 3 per cent Karo syrup, and 82 per cent tap water by weight. To this was added 5 Gm. brewer's yeast per pig per day which was graduated to 12.5 Gm. per pig per day over a 33-day period. These pigs appeared well and healthy at all times and were discharged from the experiment after being on the diet for forty-five days. The average BUN value before going on the experimental diet was 14.1. It rose to 33.1, on the fourteenth day, which was the highest mean value for this group. When discharged, the BUN was 21.8. The mean blood sugar values ranged from 106.0 prior to time of the experimental feeding to 145.5 when on this diet for twenty-one days.

Ration D.—In order to explore the effect of FFS concentration on baby pigs, 11 pigs (lot 22) were fed a diet consisting of 20 per cent FFS and 80 per cent water. These pigs were 10 days old at the start of the feeding trial and all were dead at the end of a week. In fact, many of the pigs were very sick after twenty-four hours. An analysis of their blood at that time showed a marked increase in the BUN level over what it was the day previous. The mean for the group prior to the experiment was 18.0 and one day later it was 87.1. Except for the presence of a colorless, lacelike, membranous exudate that covered the fundic region of the stomachs of several pigs in this group, there were no gross lesions observed at the postmortem examination.

The pigs that received the 30 per cent FFS diet (lot 5) were all dead in four days. They were prostrate and comatose on the day following the start of the experiment. The youngest pigs that received this diet were 11 days old and the oldest, 15 days old. The histologic changes in the kidneys and liver of the pigs in both lots 22 and 5 were those of marked degenerative processes.

The results point quite clearly to the fact that the 20 and 30 per cent FFS diets were highly lethal and caused a more acute form of

the toxemic-uremic syndrome than did the diet which carried 15 per cent FFS.

Ration F.—Having demonstrated quite conclusively that a diet containing 15 per cent or more of the spray process FFS mixed with water was highly injurious for weaned pigs under 20 days of age, the next trials were designed to learn if a diet containing a lesser proportion of FFS might produce similar results.

Lots 16, 21, and 28, containing a total of 23 pigs, were started on a ration containing 10 per cent FFS and 90 per cent tap water by weight. The survival rate of the pigs on this diet was high, but the clinical findings in all but 1 were those we would associate with chronic uremia. A single case of acute uremia occurred, which resulted in death eight days following the first feeding of this diet. A second pig came to a fatal termination after the seventeenth day of feeding. In general, the BUN values exhibited a more rapid increase than was the case with those on the 15 per cent FFS but, after about ten days on the diet, they tended to decrease. The mean BUN values during their course of chronic uremia varied between 28.0 and 35.0. During the same period, the blood sugar values varied between 95.0 and 120.0.

The results of these trials indicate that a diet composed of FFS in the proportion of 10 per cent by weight is tolerated by pigs of a week, or more, of age.

Ration G.—Since the addition of D seemed to enhance the lethal effects of the 15 per cent FFS diet, even though it widened the nutritive ratio, it was considered advisable to feed a similar mixture where the FFS was reduced to 10 per cent. Of the 17 pigs (lots 25, 27) started on this diet, 9 died during the first week of the experiment and 3 more between the ninth and twenty-third days. In all but 1 of these animals, the BUN rose to values between 35.0 and 180.0 and the blood sugar varied between 38.0 and 113.0. For the most part, the symptoms and postmortem findings simulated those associated with the acute form of the toxemia-uremia syndrome. Five pigs survived and, in general, the BUN values at no time were elevated to a significant degree.

The results strongly suggest that the addition of D to the diet supplying 10 per cent FFS was decidedly detrimental to the pigs. It provoked an acute disorder quite comparable to that which occurred when D was added to the 15 per cent FFS diet.

Lot 26, containing 7 pigs, received ration G for only two days and then switched to ration D (20% FFS). Clear water was available to them at all times. Three of the pigs died and 4 remained in reasonably good condition throughout the experimental period of forty-seven days. Two of the deaths occurred eight days from the beginning of the experiment and the other, ten days. The cause of death in these cases stems to the two-day feeding of ration G. They were very sick three days from the start of the feeding. The BUN on these pigs on the third day averaged 65.5. A record of the food and water consumption by this group indicated that these pigs tended to balance their FFS intake at approximately a 12 to 13 per cent level, although there was a wide daily fluctuation. The clinical condition and BUN values of the 4 pigs that finished the experimental trial was quite similar to those that received ration F (10% FFS).

Ration H.—Ten pigs (lot 24) were fed on a diet which contained 10 per cent FFS, 8 per cent D, and 82 per cent tap water. To this was added brewer's yeast (Y) and dried cereal grass (Cg) at the rate of 1 lb. of each per 100 lb. of the diet. There was a tendency for Y and Cg to settle out and collect on the floor of the trough, but this did not prevent their being ingested, since the trough was shallow enough to permit the pigs to eat them from the bottom.

With the exception of 1 pig (B973) that died from an acute uremia on the thirteenth day of the experimental feeding, none of the others showed an appreciable increase in the BUN levels at any time throughout the forty-six days they were on this diet. A second pig did not complete its full stay in the experiment. On the twenty-fourth day, it died from an infection of the urinary bladder and ureters.

From the results with this single lot, it would seem that Y and Cg supplied factors which prevented the occurrence of the lethal disorder that was so clearly manifested when these substances were omitted. The mechanism behind this result is not yet understood.

COMPANION GROUPS AND EFFECTS OF CHANGED RATIONS

Some groups of pigs were paired for comparative purposes and some lots were split into sublots for comparing trends in BUN values of different rations. In each case where pigs were allotted for these studies, the pigs were picked by random selection. The term of significant difference used in the following sections refers to statistical significance only.

Most of the pigs of lot 7 were morbid at 21 days of age or shortly thereafter. The pigs of lot 7c (fed CM containing 3½% butterfat for two days and then enriched to 7% for seventeen days), which were not too greatly injured by the previous ration, tended to decrease in their BUN values, while those remaining on 15 per cent FFS (lot 7a) tended to increase.

Lots 8 and 9 were pigs of the same litter. At 8 days, the groups did not differ significantly in their BUN values. At later ages, the differences were significant ($P = < 0.01$ at 13,

18, and 29 days and $P = < 0.05$ at 25 days). No difference was found in the BUN levels of lots 8b vs. 8c or 9a vs. 8b at 29 days and 46 days; however, lot 8c vs. 9a were different ($P = < 0.05$). This indicated that the BUN values are significantly greater for those pigs fed 15 per cent FFS than those fed CM and that the levels may be reversed by switching the pigs from one ration to another.

Lots 11 and 12 were composed of pigs of four litters. At average ages of 10 and 16 days, these lots did not differ significantly in their BUN quantities. Later companion analyses were not possible but, at 19 days, lot 12 (15% FFS) had risen to an average BUN level of 106, and lot 11 (3½% CM) remained at a relative low of 20 average BUN at 22 days.

After the pigs of lot 12 had died, lot 11 was split into two groups; 11a was continued on 3½ per cent CM and lot 11b was put on 15 per cent FFS ration. At 22 days, the pigs which were later put into lots 11a and 11b did not differ in their BUN levels. When 29 days old, the two groups did not differ when all pigs of lot 11a were included, but if the pig that died at 1/22 were eliminated, then a difference of $P = < 0.01$ was found in the BUN levels of lots 11a and 11b. The high mortality of lot 11b presented further comparisons.

Lots 13 and 14 were also composed of pigs from four litters. At 13 days, the lots did not differ significantly in their BUN values; they then quickly separated in a short time (± 3-5 days). After switching lot 13 from 3½ per cent CM to 15 per cent FFS, the BUN levels increased. When 44 days old, the pigs later to be placed into lots 13b and 13c did not differ significantly in BUN values. After being on 3½ per cent CM for five days, lot 13b showed a significantly lower ($P = < 0.05$) amount of BUN.

From all of the work done to date, it appears that the BUN levels of the baby pigs studied may be altered by the composition of their ration and that this change may be effected at

TABLE 2—Mean Blood Urea Nitrogen Values of Baby Pigs at Various Ages**

Lot No.	Blood Urea Nitrogen Values
7*	(21)25.4±6.76
7*a	(21)22.0±3.74 (23)25.6±7.0 (28)30.0 (33)38 (40)42 (44)45
7*b	(21)28.8±7.76
7*c	(23)2 pigs died 2/1†av. = 174 other 3 pigs av. = 39.3 (28)12.5 (33)1/11 78 other 14
9*	(8)8.3±0.69 (13)24.9±3.65 (18)29±5.2 (25)54.7±30 [(23)1/18 98 1/19 52 other 2 av. 34]
9*a	(29)41.5 (46)18.8 (55)15
12‡	(10)12.1±6.23 (16)22.0±10.45 (19)106±43
14‡	(13)5.88±1.55 (16)50.7±39 (32)44
20	(9)20
23	(9)10.5±3.0 (15)36 (18)1/8 348 other 2 av. 21 (22)30 (24)44 (29)48
10	(14)16 (18)20 (35)35 (44)31 (51)31
11	(10)9.9±3.1 (16)14.7±7.1 (22)20.2±6.9
11a	(22)19.5±4.97 (29)52.8±63 1/22 165 others av. = 25±6.09 (38)18.5±2.36
11b	(22)21.1±8.91 (29)89.35±4.5
13	(13)6.23±0.33 (25)21.88±14.84 (32)22.68±13.9
13a	(39)41.85±22.1 (44)32.43±6.46
13b	(51)23.75±3.23
13c	(51)35.59±5.35
8*	(8)9.47±4.88 (13)10.38±3.2 (18)8.22±1.89
8*a	(25)13.82±1.72 (29)11.36±4.63
8*b	(46)21 (55)18
8*c	(46)34 (55)30
16	(9)33 (16)27 (28)27 (42)22 (35)52
21	(9)17.78±3.5 (15)27.0±4.25 (20)36.3±7.07 (22)35.2±8.08 (27)33.39±6.87(33)30.4±2.93
21a	(33)30.2±2.95 (39)29.0±22.1 (47)13
21b	(33)30.4±3.2 (39)29.4±4.39 (47)36.8±13.55
28	(9)17.67±4.44 (14)34.2±6.2 (20)33.4±5.45 (26)41.0±5.59
28a	(33)38.78±8.98 (40)35.13±4.68 (47)36.13±18.87
24	(9)13.78±3.43 (15)6.55±1.78 (21)22.51±29 1/13 omitted = 13.59±7.11 (28)13.67±4.13 (34)13.95±2.22 (42)16.91±4.57 (49)15.46±1.77
25	(7)13.38±6.1 (14)33.5 (19)27.2 (25)10.9 (31)20.5 (38)20.1 (45)12.9
27	(9)17.2±6.8 (14)38.4±31.24 (20)38.3 (26)23.7 (33)15.5 (40)12.9 (47)22.1
22	(10)21.5±6.2 (13 & 14)128.78±73.04
26	(7)14.29±5.18 (12)52.43±19.45 2/6 1/8 omitted av. = 39.0 (17)44.8 (24)43.2 (29)45.4 (35)37.7 (41)36.7 (48)31.4 (55)33
5	(15)72.0

*Minnesota No. 1 pigs. †No. pigs died/no. days on ration. ‡All pigs of lot.
**BUN in mg. per 100 cc. blood ± standard deviation. (Days of age are in parentheses.)

any age prior to 56 days, the limit of this study.

BLOOD UREA NITROGEN VALUES IN RELATION TO DAY OF DEATH

The blood urea nitrogen values in milligrams per 100 cc. of blood were arranged in relation to the day of death of the pigs, regardless of the age of the animals or the length of time they had been on their ration. The averages for the various days are as follows:* (0) 159 ± 93, (1) 99 ± 57, (2) 51 ± 25, (3) 34 ± 30, (4) 24 ± 20, (5) 27±19, (6) 17, (7) 24, (8) 24, (9) 16, (10) 22, (11) 26, (12) 27, (13) 20, (14) 17, (15) 26, (17) 14. The average for day (0) was significantly higher ($P = < 0.05$) than day (1) as was the difference between days (1) and (2). A highly significant difference ($P = < 0.01$) was found between days (0) and (2).

Thirty-eight pigs were brought to the laboratory after being with their dams for six to ten days. These pigs were bled upon arrival from the sow. Their average BUN values were 15 ± 5.6.

STUDIES OF BLOOD SUGAR LEVELS

Hypoglycemia was not a characteristic of the phenomena observed in this syndrome. Blood sugar levels varied within normal ranges. A slight increase was noted, however, near the day of death but this increase was not significant. At the day of death and earlier, the average blood sugar levels were:* (0) 130 ± 88, (1) 137 ± 51, (2) 121 ± 59, (4) 119 ± 23, (5) 115, (6) 111, (7) 122, (8) 111, (9) 99.

DISCUSSION

Very early in the course of this study, it was evident that the basal diet (15% FFS) did not support the anticipated growth and development of the pigs. In fact, it proved to be quite the opposite. A high proportion of the pigs receiving the basal diet developed a severe metabolic disorder which resulted in their death. The study might have been discontinued or considerably modified if it had not been found that the clinical, blood chemistry, and necropsy findings on the sick and dead pigs simulated to a marked degree many of the clinical, blood chemistry, and necropsy findings which the authors have associated with a naturally occurring disease of suckling pigs.

Qualitatively, the basal diet approximated fresh cow's skimmilk and it also approximated the fat-free solids of sow's milk. When the FFS was increased to 20 per cent or to 30 per cent, the toxicity effects of the diet were increased very decidedly. The BUN values rose rapidly and to a high level, the malaise was more profound, and the course of the disorder markedly shortened. When the FFS was decreased to 10 per cent, the disorder was apparent; although the pigs lived, they ran a clinical course of a more chronic nature.

The caloric intake of the basal diet and also the diet containing 10 per cent FFS was stepped up by the addition of D. This had a pronounced harmful effect on most of the pigs receiving the added D. The blood nitrogen retention rose to exceptionally high levels with early fatalities.

When Cg and Y were included in the diet, the marked toxicity effects appeared to be alleviated. It is not clear why the added Y and Cg appeared to be so effective in preventing the acute toxic effect of this diet. Under normal circumstances, it is difficult to conceive that a vitamin deficiency would arise so rapidly as to cause death in a matter of a few days, as did occur with some of the diets that were fed.

The clinical symptoms and pathologic findings combined to form a syndrome having the characteristic of an intoxication. Muscular weakness, muscular twitchings, nausea and vomiting, periodic breathing, at first shallow and then deep respiratory movements (Cheyne-Stokes breathing), and coma were outstanding physical signs, while the principal tissue changes were those of degeneration. This was very marked in the kidney and no doubt was the basis for the increase in the BUN.

A suggestion of the possible cause for the effects of skimmilk diets observed in these studies is given in the reports on the utilization of galactose and lactose by albino rats. Guha[7] reported that rats placed on a diet containing galactose as the sole carbohydrate rapidly decreased in body weight and died. Death could be prevented by replacing part of the diet with glucose or by fat but not by increasing the protein of the diet.

Schantz, Elvehjem, and Hart[8] studied the relation of fat to the utilization of lactose in milk. Rats which received fresh whole milk utilized the lactose in the milk very completely, as indicated by negative tests on the urine for reducing substances. Rats on skimmilk plus a mineral supplement and carotene for a few days gave positive tests for reducing substances in the urine. They found that the addition of butterfat, corn oil, lard, cocoanut oil, linseed oil, or palmitic or oleic acids would prevent the loss of sugar in the urine. In a similar study on a pig and a calf, they found complete utilization of the sugar on a whole milk diet. However, after two weeks on skimmilk, con-

*(0) = day of death, (1) = day before death, etc.
*(0) = day of death, (1) = day before death, etc.

siderable amounts appeared in the urine. A more recent report from the same laboratory[9] reported that albino rats on a diet of skimmilk plus minerals excreted 23 to 27 per cent of the ingested galactose. When 4 per cent butterfat was added to the diet, the galactose excretion decreased to 2.9 to 3.8 per cent.

Other than the suggestions which came from the studies of the effect of high galactose or skimmilk diets with rats, there are no definite indications as to the specific causative factor or factors responsible for the disorder as observed in the pigs receiving these diets. The data are presented without any attempt to define the etiology of the apparent toxic nature of this diet.

SUMMARY

1) Reconstituted fat-free skimmilk diets at levels ranging from 10 per cent to 30 per cent and supplemented with minerals and vitamin A appeared to be toxic to young suckling pigs 7 to 14 days old. The toxic effects were manifested by high morbidity and mortality with both gross and microscopic evidences of degenerative changes in the kidney, liver, and adrenals, in particular, and with high blood urea nitrogen values.

2) The addition of dextrin to the diet accentuated the apparent toxic effects of the reconstituted fat-free skimmilk diets.

3) The addition of dehydrated cereal grass and yeast seemed to alleviate the apparent toxic effects.

4) The specific etiologic factor or factors responsible for the disorder are not known.

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The Role of Plasma Fractions in Passive Immunization and Treatment

H. S. CAMERON, Ph.D., M.S., D.V.M.

Davis, California

THE PRESENCE of Brucella agglutinins and Staphylococcus antitoxin has been demonstrated by Cameron[1] in fraction II from normal bovine and porcine plasma. The fraction from swine also protected pigs against a strain of hog-cholera virus, and mice against a lethal inoculation of *Erysipelothrix rhusiopathiae*.[2] The purpose of this paper is to report further investigations on the antibody content of the fraction against other organisms and another strain of hog-cholera virus.

The characteristics of the fractions have been reported by Cohn and his associates,[3] under whose method fractionation was performed by the Armour Laboratories. This report will deal only with fraction II, since immune bodies are concentrated chiefly in this fraction. The material used was in a sterile solution containing 16.5 per cent protein in 100 cc. of 0.3 m. glycine with no preservative. The blood from which the fraction was obtained represented a pooled sample from about 400 hogs, cattle, or sheep, without regard to antemortem or postmortem examination.

Hog Cholera.—Because, in the original investigations,[1] highly favorable results were obtained in this disease, it was advisable to repeat the work under field conditions with another strain of virus. Facilities for a field test were made available in a large herd where exposure to virus was heavy and where simultaneous vaccination at an early age was practiced. Each of 40 pigs, ranging in age from 4 to 6 weeks, was given 4 cc. of a commercially obtained strain of hog-cholera virus. Of these, 30 pigs were given hyperimmune serum, and the remainder, 10 cc. of the *gamma* globulin solution. This amount of the fraction was more than double that found necessary to protect pigs in the original experiment. The results did not conform with the original test since all 10 pigs died from cholera, although the disease exhibited a more chronic course.

Because of these findings, the work was again repeated, under well-controlled conditions, using still another strain of virus of known high virulence. Thirty-one pigs were divided into four groups of 10, 10, 6, and 5 pigs. Each received 2 cc. of virus. Groups 1 and 2 received 10 cc. and 5 cc., respectively, of porcine *gamma* globulin. In group 1, out of 10 pigs inoculated, 1 died on the tenth day, 2 on the eleventh, and 4 on the thirteenth. The remaining 3 survived. In group 2, receiving 5 cc. of *gamma* globulin, 2 died the eighth day, 2 the eleventh, 1 the twelfth, and the remaining 5 the thirteenth. In group 3 (virus alone), all pigs died between the seventh and twelfth days. In group 4 (bovine *gamma* globulin, 10 cc.), the first death was on the tenth day, and the fifth and last on the thirteenth.

TABLE 1—Mouse Protection Against Pasteurella Multocida

Group	No.	Inoculum*	Results
1	31 mice	*P. multocida* control	All dead within 48 hr.
2	100 mice	*P. multocida* and bovine γ globulin	79 survived. 21 died within 60 hr.
3	40 mice	*P. multocida* and ovine γ globulin	33 dead in 48 hr. Only 3 survived 6 days.

P. multocida suspension 6 billion per cc., 0.5 cc. intraperitoneal *gamma* globulin solution, 0.5 cc. intraperitoneal.

From these results, it would appear that the porcine *gamma* globulin, while having some antagonism to virus, did not have immunizing properties that could replace hyperimmune serum.

Pasteurella Multocida.—Because of its cosmopolitan distribution, its pathogenicity for laboratory animals, and its rôle of opportunist in diseases of domestic livestock, the bovine fraction was tested for antibodies against this organism. Two strains were used, one isolated from a rabbit, the other from a chicken. Mouse protection was the method adopted in the assay. The inoculum consisted, in all cases, of 0.5 cc. of a suspension containing 6 billion organisms per cc. Intraperitoneal injections were used in 126 mice, 26 of which received the organism alone. The remaining 100 received, in addition to the organism, 0.5 cc. of bovine *gamma* globulin solution. The tests were run in series, but since there was no significant difference between the various series, and since no difference was apparent between

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From the Department of Veterinary Science, University of California, Davis.

The fractions were made available through the courtesy of Armour and Company.

the strains, the results can be summarized as one group, in addition to the controls.

All control animals died in from twenty-four to forty-eight hours. Of the 100 protected mice, 21 failed to survive for more than sixty hours. The remaining 79 all showed symptoms of distress, but recovered and were still alive twenty days following the tests, when they were disposed of.

TABLE 2—Mouse Protection Against Salmonella Choleraesuis

Group	No.	Inoculum*	Results
1	15 mice	*S. choleraesuis* control	All dead within 36 hr.
2	80 mice	*S. choleraesuis* and porcine γ globulin	46 survived. 34 died in 6 days when test ended.
3	50 mice	*S. choleraesuis* and bovine γ globulin	2 survived. 48 died within 6 days when test ended.

*S. *choleraesuis* suspension, 0.5 cc. Gates 3 intraperitoneal *gamma* globulin solution, 0.5 cc. intraperitoneal.

In another series, 40 mice were injected with a similar bacterial suspension simultaneously with ovine *gamma* globulin. Ten were left as unprotected controls. At the end of twenty-four hours, 3 of the 40 were dead; in forty-eight hours, 33. At the end of six days, only 3 were alive. All controls were dead in forty-eight hours. Apparently, the protection afforded by the fraction originating from sheep plasma did not have an immunizing value comparable to that from cattle.

Clostridium Welchii Type D Toxin.—Sheep plasma fraction II was tested for antitoxin content. This experiment was conducted because of the prevailing opinion of a number of workers that enterotoxemia (pulpy kidney disease) of sheep is primarily a toxemia due to the absorption of this specific toxin formed in the digestive tract, and that it can be controlled by treatment with the specific antitoxin. A potent toxin, which killed mice within six hours, was prepared. In addition to toxin, one group of mice received ovine *gamma* globulin, another group, specific antitoxin (horse serum) prepared commercially. The third group received toxin alone. The group receiving commercial antitoxin was completely protected; no protection was afforded by the ovine *gamma* globulin.

Salmonella Choleraesuis.—Because of its widespread distribution in swine, tests were made to compare the immunizing value of porcine and bovine *gamma* globulin against this organism. Eighty mice were injected intraperitoneally with 0.5 cc. of a Gates 3 suspension plus 0.5 cc. of the solution of porcine *gamma* globulin. Ten were given the organism alone. All controls died in thirty-six hours, while in the protected group, 46 lived and 36 died over a period of six days.

Using the bovine fraction, 10 controls were dead in forty-eight hours, while out of 50 immunized mice, 48 were dead in six days.

Brucella Suis.—Although passive immunization has not, as yet, an application in brucellosis, the finding of a strain of *Br. suis* which invariably proved fatal to mice within forty-eight hours prompted an experiment to test the effect of the bovine and porcine fractions on the strain. Three groups of mice were all inoculated with 0.5 cc. of a suspension of *Br. suis* SSH 138 containing approximately 2 billion organisms. Group 1 (20 mice) received, in addition, 0.5 cc. of the solution of the porcine fraction; group 2 (20 mice), a similar amount of the bovine fraction. Group 3 (10 mice) remained as controls. All controls died within forty-eight hours. Of the 20 receiving the porcine fraction, 10 were alive six days following inoculation, when they were destroyed. In the group receiving the bovine fraction, 15 out of 20 were dead in six days.

DISCUSSION

The significant feature of these tests is the indicated possibility of the use of the fraction in low grade infections rather than in acute infections such as hog cholera. On the other hand, the results with *Ery. rhusiopathiae* in mice suggest a value in the acute form of this infection. In the case of hog cholera, the variation in the results of the three tests can only be interpreted in terms of the virulence of the virus used. It is apparent, therefore, that normal plasma fraction cannot, at present, supplant hyperimmune serum. The *P. multocida* tests are very significant. The test was severe, and protection, under the circumstances, was such that field trials are warranted. It is somewhat surprising that comparable results were not obtained with the ovine fraction. Possibly the plasma was obtained from young lambs that had not developed sufficient antibody content. Since lambs are often marketed very young, it is doubtful if the fraction would be rich enough in anti-

TABLE 3—Mouse Protection Against Brucella Suis

Group	No.	Inoculum*	Results
1	10 mice	*Br. suis* control	All dead in 48 hr.
2	20 mice	*Br. suis* and porcine γ globulin	10 survived, 10 dead at 6 days when test ended.
3	20 mice	*Br. suis* and bovine γ globulin	5 survived, 15 dead at 6 days when test ended.

*Br. suis suspension, 0.5 cc. SSH 138, 2 billion per cc. intraperitoneal *gamma* globulin solution, 0.5 cc. intraperitoneal.

body to be of value in this field, unless obtained from older animals. The tests with *Cl. welchii*, which is omnipresent in the intestinal tract where it produces a toxin, were conducted not from the standpoint of potential prophylaxis but rather to ascertain if a detectable amount of toxin was absorbed. No antitoxin could be demonstrated in the fraction when compared to commercial antitoxin. Likewise, with the other cosmopolitan organism, *S. choleraesuis*, adequate mouse protection was provided, and again field trials are justified.

The tests on mouse protection against a strain of *Br. suis* are significant since a septicemia was overcome in an appreciable number of animals. The possibility of similar protection in acute brucellosis of swine is suggested from these tests, but since pathogenesis is usually advanced before symptoms become apparent, little hope is expected from passive immunization. The tests, nevertheless, will be conducted on swine.

Should *gamma* globulin eventually find a rôle in disease control, it is essential that the fraction come from stock from an area where intensive livestock raising is conducted. An examination of the fraction from different areas would undoubtedly show a geographic variation in antibody content against certain antigens. This variable would be overcome by the pooling of the plasma of a large number of animals, preferably from large feed yards.

In addition to fraction II, fraction V (serum albumin) has a potential use in shock treatment and maintaining blood volume, thus combating dehydration. Its use in shock therapy in dogs has been suggested in a preliminary report by Brasmer, Taylor, and Stephenson.[4] Fraction V can be used on a heterologous species, being only slightly antigenic. Fraction II, on the other hand, should only be employed on the homologous species because it is highly antigenic.

Fraction I is fibrinogenic and nonantigenic and is employed where clot formation is desired.

This report cannot, of course, deal with comparative costs, such as comparing the cost of producing the fractions with that of antiserum. Much will depend on how many of the intermediate fractions can be used. It is not expected that normal *gamma* globulin will have more immunizing properties than immune serum. If any advantage is to be gained, it must be in the cost of production. If that can be markedly lowered, plasma fractions have a place in the control of livestock diseases.

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DISCUSSION

DR. A. F. SCHALK (Columbus, Ohio): I did not understand the fraction II and V, and what you indicated about homologous parts.

DR. CAMERON (Davis, Calif.): Fraction V serum albumin is not antigenic and can be used on heterologous species. In fact, I have reports on considerable use of bovine serum albumin in mares and foals, and I have only one report of any anaphylactic shock. On the contrary, fraction II *gamma* globulin is highly antigenic and should be used only on homologous species.

Susceptibility of Furbearing Animals to the Toxins of *Clostridium Botulinum* Types A, B, C, and E

E. R. QUORTRUP, D.V.M., and J. R. GORHAM, D.V.M., M.S.

Pullman, Washington

LITERATURE

The first authentic outbreak of botulism in mink was reported in 1938 by Hall and Stiles.[1] This outbreak involved a loss of 146 out of 148 animals (96%). The causative organism was described as *Bacillus botulinus* type A. Examination of suspected foods did not reveal the source of the organism, but toxin was demonstrated in filtrates of scrapings from feeding boards, of the stomachs of dead mink, and of their feces. It is not known how many samples were typed, and there is a possibility that other strains may have been present.

In August, 1940, Quortrup and Holt[2] reported an outbreak in mink occurring in Utah in October of the preceding year. The causative organism in this case was *Clostridium botulinum* type C. Three ranches having a common source of feed, in which a dead calf had been incorporated, were involved in this outbreak. On one ranch, 461 mink, out of a total of approximately 500, died within two or three days.

In September, 1940, in Wisconsin, Chaddock[3] described a characteristic outbreak of botulism in mink. This outbreak was caused by contaminated beef and horse meat, which was distributed to several ranches, resulting in severe losses on all of them. He stated that *botulinum* antitoxin was used, but did not mention the type. The fact that mink experimentally fed with the toxin died in fifteen hours suggests that type C might have been involved, as we have not seen any other type act so rapidly.

Although numerous reports of botulism in mink have appeared in the literature in recent years, no mention of the specific type involved can be found except in the two cases cited.[1, 2] McDermid[4] wrote in 1946: "The types which we believe concern us are mainly types A and B, and some of us feel that type C is involved"; but he did not elaborate on the subject, and it is not known if they were typed. Botulism in mink has also been reported in Canada by Kennedy[5] and others.

In most cases, losses on individual ranches have been severe. In a trade journal, one rancher in Minnesota reportedly lost $75,000 worth of fur due to this disease. At times, there was not sufficient antitoxin available in the entire United States to treat the number of mink affected in an individual outbreak. This prompted the United Mink Producers Association and the Great Lakes Mink Association to establish a *botulinus* antitoxin "bank,"[6] carrying enough antitoxin to treat 16,000 animals.

Impressed with the seriousness of the matter, the writers undertook a study to determine susceptibility and type specificity of *Cl. botulinum* on various fur animals, so that recommendations might be made in regard to appropriate treatment and prophylaxis.

EXPERIMENTAL

Experiment 1.—Preliminary experiments were undertaken in the fall of 1947 to determine the toxicity of various *botulinum* cultures at hand. Unfortunately, our entire mouse colony was lost due to an outbreak of paratyphoid, so our cultures could not be titrated prior to feeding them to mink. For that reason, the first experiment will only be mentioned briefly here. However, it was found that none of five A strains administered to 14 mink and 1 ferret were toxic to these animals when fed in amounts of 10 cc. of culture.

Six type B cultures were fed to as many mink with the result that 2 died with delayed action in from sixty to 168 hours.

All type C cultures carried in stock, as well as those acquired from other laboratories, were completely nontoxic to mink as well as mice, whereas all recently isolated cultures were rapidly fatal.

It has repeatedly been our experience, and that of others, that cultures of *Cl. botulinum* type C lose their toxicity soon after isolation.

Experiment 2.—When mice became available

From the Fur Animal Disease Research Laboratory, U. S. Bureau of Animal Industry, in collaboration with the Division of Veterinary Science, Agricultural Experiment Station, Washington State College, Pullman.

Dr. Quortrup at present is on leave of absence with the Department of Veterinary Science, University of California, Berkeley.

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Appreciation is expressed to Dr. K. F. Meyer of the George Hooper Foundation, San Francisco; Dr. L. R. Vawter of the University of Nevada; Jensen-Salsbery Laboratories, Kansas City, Mo.; U. S. Public Health Service; American Type Culture Collection; Dr. G. M. Dack of the University of Chicago; and Dr. C. E. Dolman of the University of British Columbia for furnishing some of the cultures used in the experiments. We are also indebted to Mr. Ralph Hervey, bacteriologist, Bear River Refuge, Utah, for collecting and sending us the duck stomachs from which we isolated type C cultures. Appreciation is also expressed to the U. S. Fish and Wildlife Service for technical knowledge gained, during the years Dr. Quortrup worked for that agency, which facilitated the present study.

for toxicity determination, a more elaborate experiment was conducted (*see* table 1) and the mouse m.l.d.'s of the various cultures were determined before the mink were challenged. Before the toxin was administered to mink and ferrets, they were fed lightly the preceding day to insure a good appetite when the test dose was given. The toxin was mixed into their regular ration. In spite of the obnoxious odors emanating from the cultures, the animals showed no reluctance in eating the feed, which in every instance was devoured completely.

Type A.—A total of ten strains of type A were tested by feeding identical cultures to 1 mink and 1 ferret. These ranged in toxicity from 500,000 to over 10,000,000 mouse m.l.d.'s per cubic centimeter. The dosage fed to mink and ferrets is indicated in table 1. Using such enormous amounts of toxin, it would be expected that all the exposed animals would die if they were susceptible at all, but this was not the case. The results were that 5 out of 10 mink and 3 out of 10 ferrets, having ingested from 25,000,000 to 60,000,000 mouse m.l.d.'s, died in from sixty to 216 hours. This extremely slow action and high dosage necessary to cause illness would indicate a high resistance of mink to type A toxin. Five mink and 6 ferrets remained unaffected and 1 ferret became sick but recovered spontaneously. In other words, 50 per cent of the strains used were lethal to mink but only 30 per cent to ferrets. Admittedly, it would have been better to have used several mink for each strain tested, but the ferret was supposed to serve as a duplicate, making the experiment less costly. As it is, about 80 mink and ferrets were sacrificed, which represents no small investment; and considering the number of strains used, we feel that our results are a fairly accurate index to the situation.

Type B.—Eight strains of type B were used, only two of which were lethal to mink, one acting rapidly causing death in thirty hours and one with a delayed action of eleven days. Dack[7] has described delayed action of *Cl. botulinum* toxin in human beings, and this has also been observed by the writer in waterfowl. Two ferrets were killed by B cultures in from forty-four to forty-five hours. The mortality for both mink and ferrets was 2 out of 8 (25%). The mouse m.l.d. of the B strains varied from 5,000 to 10,000,000 per cubic centimeter. From 6,000,000 to 25,000,000 mouse m.l.d.'s were required to produce lethal effects in mink.

Type C.—As mentioned previously, no toxic C strains could be obtained from other laboratories, so five strains of this type were isolated by the authors from stomachs of ducks obtained from the Bear River marshes in Utah. These were typed with antitoxin obtained from the United States Public Health Service. As

TABLE 1—Results of Feeding Tests of Mink and Ferrets, Using Titrated Cultures

Cl. botulinum Strain (No.)	Source	Type	Mouse m.l.d. per cc.	Mink: Mouse m.l.d.'s in feed	Mink: Results (hr.)	Ferret: Mouse m.l.d.'s in feed	Ferret: Results (hr.)
7948	A.T.C.C.	A	10,000,000	55,000,000	.....	55,000,000	XXX 101 hr.
782	Quortrup	A	10,000,000	55,000,000	.....	55,000,000	.....
28	U.S.P.H.S.	A	10,000,000	60,000,000	XXX 97 hr.	60,000,000	.....
20	Jen Sal	A	10,000,000	60,000,000	XXX 192 hr.	60,000,000	XXX 92 hr.
33236	Vawter	A	10,000,000	45,000,000	XXX 68 hr.	45,000,000	.....
109	Dack	A	10,000,000	60,000,000	.....	60,000,000	XXX 120 hr.
62	Dack	A	10,000,000	60,000,000	XXX 60 hr.	60,000,000	X 96 hr.
56	Meyer	A	5,000,000	25,000,000	XXX 216 hr.	25,000,000	.....
54	Gorham	A	1,000,000	5,000,000	.....	5,000,000	.....
172	Meyer	A	500,000	2,500,000	.....	2,500,000	.....
29	U.S.P.H.S.	B	10,000,000	55,000,000	.....	55,000,000	XXX 45 hr.
113	Meyer	B	5,000,000	25,000,000	XXX 264 hr.	25,000,000	.....
7949	A.T.C.C.	B	100,000	600,000	.....	600,000	.....
21	Jen Sal	B	100,000	600,000	.....	600,000	.....
39	Meyer	B	20,000	100,000	.....	100,000	.....
169	Meyer	B	5,000	25,000	.....	25,000	.....
	Dack	B	1,000,000	6,000,000	XXX 30 hr.	6,000,000	XXX 44 hr.
169	Dack	B	50,000	300,000	.....	300,000	.....
37	Quortrup	C	10,000,000	50,000,000	XXX 18 hr.	50,000,000	XXX 78 hr.
48	Quortrup	C	1,000,000	5,000,000	XXX 18 hr.	5,000,000	XXX 21 hr.
50	Quortrup	C	1,000,000	5,500,000	XXX 18 hr.	5,500,000	XXX 32 hr.
47	Quortrup	C	500,000	3,000,000	XXX 18 hr.	3,000,000	XXX 27½ hr.
49	Quortrup	C	200,000	1,200,000	XXX 33 hr.	1,200,000	XX Recovered
Nanaimo	Dolman	E	2,000	20,000	.....	20,000	.....
35396	Hazen	E	3,000	30,000	.....	30,000	.....

XXX = Lethal. XX = Very sick. X = Slightly sick. ..... = Negative.
All exposed animals were kept under observation for at least fourteen days.

shown in table 1, the mortality of mink exposed to the toxin was 100 per cent, and the action was very rapid, requiring from less than eighteen hours to a maximum of thirty-three hours to kill. Four out of the 5 ferrets exposed died in from twenty-seven and a half to seventy-eight hours after ingestion, and 1 was near death but recovered. There was no natural immunity in this animal, as a second exposure thirty days after recovery, resulted in death thirty hours after challenge with another strain.

The sudden onset and high mortality, which is characteristic of outbreaks on many ranches, would indicate that type C is the principal offender. Chaddock,[8] in a recent communication, states that most of the cases he has dealt with have been of the C strain. He calls our attention to the loss of 6,000 mink in a very short time from the feeding of frog legs that had been condemned for human consumption. Furthermore, he states that other outbreaks he has observed, in almost every case, have been from the feeding of tainted fish. It is not known if the organism involved in these outbreaks were typed.

An attempt to determine the mink m.l.d. on one of our C strains was made, and is described in table 2. It was found that this was 0.01 cc. of culture, equaling 100,000 mouse m.l.d.'s. Based on the limited data at hand,

TABLE 2—Determination of M.L.D. of Clostridium Botulinum Type C on Mink

Strain	Mouse m.l.d. (*per os*)		Results
50	10,000,000	1 cc.	XXX in less than 18 hr.
50	5,000,000	0.5 cc.	XXX in less than 18 hr.
50	1,000,000	0.1 cc.	XXX in less than 18 hr.
50	200,000	0.02 cc.	XXX in less than 20 hr.
50	100,000	0.01 cc.	XXX in less than 44 hr.
50	50,000	0.05 cc.	
50	20,000	0.002 cc.	
50	10,000	0.001 cc.	

The mink m.l.d. of this culture appears to lie between 50,000 and 100,000 mouse m.l.d.'s.
Note that the mink m.l.d. is based on feeding, while the mouse m.l.d. is based on intraperitoneal injections.

this would indicate that type C is 250 times more toxic to mink than type A and 60 times more toxic than B. With such a small amount being lethal to mink, it is easy to understand how a ranch can lose all its animals in a short time, if contaminated feed is used.

Type E.—Two cultures of *Cl. botulinum* type E were obtained from Dolman,[9] one isolated by himself from salmon, and one isolated by Hazen[10] from German-canned sprats. None of these strains produced any ill effects in either mink or ferrets. Table 1 shows that they possess a relatively low toxicity for mice, but as stated by Dolman, "The mouse-killing power of type E *botulinus* toxin may be a poor index of its lethal power for man." This, perhaps, may also apply to mink. The number of E strains tested is obviously inadequate for final conclusion, but more will be tested if they become available.

Susceptibility of Foxes.—As dogs are highly resistant to botulism, susceptibility of foxes was not expected, and no natural outbreaks have been reported. But, in order to settle the question, the following experiment was undertaken in Silver Foxes.

Cl. botulinum Strain (No.)	Type	Mouse m.l.d.'s in feed	Fox (No.)	Results	Fox (No.)	Results
782	A	70,000,000	1	neg.	2	neg.
29	B	100,000,000	3	neg.	4	neg.
50	C	50,000,000	5	neg.	6	neg.

These foxes were kept under observation daily for one month and never showed any symptoms. In view of the enormous doses of toxin fed, it is concluded that foxes are immune.

Susceptibility of Other Fur Animals.—When previously working for the U. S. Fish and Wildlife Service, Dr. Quortrup found that coyotes and wildcats *(Lynx rufus)* were not susceptible to *Cl. botulinum* types A and C. Type B was not tried. Muskrats were found to be susceptible to type C; the two other types were not tested.

Symptomatology of Mink and Ferrets.—In many cases, no symptoms were observed. The mink would be well in the evening and dead the next morning. It was not uncommon to see an animal apparently well, and find it dead two hours later. In the cases where a definite disease syndrome was observed, it was characterized by progressive paralysis and dyspnea. In one instance, a ferret recoverd spontaneously after having reached a stage of complete prostration. Due to the anatomic difference, paralysis of the nictitating membrane is not as readily observed in mink as it is in birds, but it does occur. In the terminal stages, there are usually convulsions, and the animal froths from the mouth. No constant postmortem lesions were found, although hyperemia of the lungs was observed, especially if the animal had showed a severe dyspnea before death.

DISCUSSION

The reason C strains lose their toxicity rapidly after isolation is not known, and this fact hampers laboratory work consid-

erably. It appears that it would be a worthy project to attempt to develop a medium which would maintain the toxicity of this organism indefinitely.

Considering the severe losses of mink on many ranches during the past few years, the practical aspects of the application of *botulinus* toxoid should be investigated. Nigg and co-workers[11] have described a method of production of alum-precipitated *botulinum* toxoid for types A and B. This method might logically be applied to type C with equally good results. If annual vaccination with toxoid could be administered simultaneously with distemper vaccination, this would probably eliminate future severe losses due to this type of food poisoning.

SUMMARY

It was found that mink and ferrets were moderately susceptible to the toxins of *Clostridium botulinum* types A and B, and highly susceptible to type C; whereas, they appeared to be immune to type E. As little as 0.01 cc. or 100,000 mouse m.l.d.'s of type C was sufficient to kill a mink. Sometimes 55,000,000 mouse m.l.d.'s of types A and B had no effect on mink and ferrets.

It is recommended that antitoxin containing types A, B, and C should be used in treatment of botulism in mink and ferrets.

A study to determine the practical aspects of toxoid vaccination is advocated.

Foxes, coyotes, and wildcats (*Lynx rufus*) were found to be immune to *botulinus* toxin. Mink, ferrets, and muskrats were found to be susceptible.

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DISCUSSION

CHAIRMAN MURPHY: Thank you, Dr. Quortrup. Now, any questions from the floor, please?

VOICE FROM FLOOR: I'd like to ask Dr. Quortrup if, with a bigger dosage, there would be immunity?

DR. QUORTRUP: There is no immunity produced.

VOICE FROM FLOOR: Would there be any way of stating how much toxin it would take to kill a man in proportion to these?

DR. QUORTRUP: I would not be able to answer that.

DR. F. B. PULLING (California): Did you attempt the diagnosis of botulism against a known antitoxin?

DR. QUORTRUP: Yes, we have demonstrated and typed the toxin in the blood stream of living, not dead, animals by serum neutralization with antitoxin.

DR. PULLING: In recovered animals?

DR. QUORTRUP: There is no demonstrable toxin left in the blood stream sixty to seventy hours following ingestion, and animals are not immune to repeated exposures. I have frequently seen, particularly among ducks, the same individual come in for treatment 2 or even 3 times within the same season.

Morphogenesis of the Thyroid Glands of Chicken Embryos

W. G. VENZKE, D.V.M., M.S., Ph.D.

Columbus, Ohio

ACCORDING TO the view held by Remak,[6] Mall,[4] Verdun,[12] and Marine,[6] the thyroid glands of the chicken embryo develop from a single median entodermal anlage. The age at which the thyroid anlage first appears and loses pharyngeal connection has been observed by Lillie,[3] Bradway,[1] and Yoshikawa.[13]

The age at which the thyroid glands become completely paired varies, according to the reports of Verdun,[12] Lillie,[3] Bradway,[1] and Yoshikawa.[13]

Bradway[1] and Yoshikawa[13] reported on the time at which nonfollicular spaces begin to form in the developing thyroid glands.

The time at which vesicles begin to form and primary follicles appear in the developing thyroid glands has been observed by Sun,[8] Bradway,[1] Yoshikawa,[13] and Hopkins.[2] These same authors have described the character of the colloid present in the developing gland and follicles.

Growth in size of the thyroid follicles and the way in which the follicles increase in number has been discussed by Hopkins[2] and Bradway.[1]

Sun[8] made iodine determinations as soon as the vesicles appeared. He found that the iodine content in the glands increases with the age of incubation. Sun[8] stated further that the morphologic point of view does not exactly agree with the chemical determinations as to when the thyroid begins to function. The percentage of active principle of the thyroid reaches a normal level immediately after hatching.

MATERIALS AND METHODS

All embryos and chicks used in this study were of a pure, single comb, White Leghorn stock maintained by the Department of Poultry Husbandry at Iowa State College. Throughout the study, the fertile eggs were obtained from the same group of hens.

A series of 420 embryos, ranging from 12 hours of incubation to hatching, was used for the morphologic studies.

The thyroid glands were fixed in either Zenker's, Bouin's, or Susa's fluid. All tissues were embedded in Altman's paraffin mixture. The sections were cut at 6 to 8 μ in thickness.

The stains most frequently employed were hematoxylin and eosin. For differentiating the connective tissue in the thyroid glands, the Zenker fixed material was stained with Mallory's connective tissue stain.

A filar micrometer was used in measuring the follicular diameters and epithelial heights. The follicular diameter was obtained by measuring the distance from basement membrane to basement membrane. In order to get a random sample of the follicular diameters, all follicles in three representative fields on every third section were measured from both the right and left gland. The number of follicles measured in each gland varied somewhat because of differences in size of thyroid glands, and variations in the plane of sectioning. However, the number of follicles measured in all cases represented a good random sample. The follicular diameters for both males and females were pooled for each interval studied. The data were evaluated in terms of the mean and standard deviation for each interval.

OBSERVATIONS

Thyroid glands arise as spherical evagination of epithelium from the floor of the pharynx. The evagination occurs between, and a little in front of, the ventral ends of the second pair of visceral pouches. This evagination first appears after 30 to 36 hours of development, and is represented by a well-defined plate of high columnar cells. At 72 hours of incubation, the plate of high columnar cells forms a saucer-shaped depression (fig. 1). At 84 hours of development, the plate resembles a well-developed sac with a rather wide mouth, and the thyroid primordium appears as a small, spherical vesicle lying beneath the floor of the pharynx. By the fourth day, the thyroid sac loses all connection with the pharyngeal epithelium.

The thyroid tissue measures 150 μ through its greatest diameter at 110 hours, and there is a slight groove in the structure both dorsally and ventrally. The nuclei of the cells about the periphery of the cellular mass are larger than those located centrally. Most mitotic figures are found near the periphery. A large number of small nuclei are found centrally in the gland tissues. There is a clear protoplasmic zone between the central and peripheral layers of nuclei (fig. 2).

This is a portion of the data presented in a thesis submitted to the graduate faculty of Iowa State College in partial fulfillment of the requirements for the Ph. D. degree, March, 1942.

Associate professor, Department of Medicine, College of Veterinary Medicine, The Ohio State University, Columbus.

Sincere thanks are extended to Dr. H. L. Foust, Department of Veterinary Anatomy, Iowa State College, Ames, for his counsel and generosity in supplying materials and laboratory equipment; to Dr. M. Lois Calhoun, Department of Veterinary Anatomy, Michigan State College, East Lansing, for her assistance in preparing photomicrographs.

At 120 hours of development, the thyroid gland tissue has become bilobed (fig. 3). The nuclei of the cells near the periphery are larger than those near the center of each lobe. Most of the mitotic figures are seen in the clear protoplasmic zone described for the 110-hour chick. A slight projection of cells can be seen on each lobe at the point where they finally separated. The two lobes are separated by a distance of 50 μ, and each measures approximately 160 μ through its greatest diameter. A single, cell-layer-connective-tissue capsule is present about each lobe. The two lobes are located dorsally and medially to the second aortic arches.

The thyroid lobes at 134 hours consist of two different types of cells. One is a small cell with little cytoplasm and a dark-straining nucleus; the other is larger, contains much clear cytoplasm, and posseses a large nucleus with little chromatin material. Mitotic figures are most prevalent among these large, clear cells. The average diameter of the two lobes through the greatest diameter is 250 μ.

Clearly defined, nonfollicular spaces can be observed at 148 hours (fig. 4). Some of these spaces are spheroidal; others are irregular in shape. These spaces do not communicate with each other but they indicate the beginning of the process of coalescence. Cells destined to become the parathyroid primordium are becoming detached from the thyroid primordium.

In the 165-hour chick, the nonglandular mesenchyme invades the nonfollicular spaces from the surface of the gland. As a result of this mesenchymal invasion, the thyroid cells are grouped into cords (fig. 5).

The ultimobranchial bodies at 177 hours are located ventral to the jugular veins, in contact with the thyroid glands. At 206 hours, the ultimobranchial bodies are partially surrounded by the thyroid glands.

Prior to 191 hours, the thyroid cells have had no special arrangement. At this time, the coiled cellular cords of the thyroid are composed of a double thickness of clearly outlined columnar cells.

At 213 hours, the thyroid gland has increased to 300 μ, measured through the greatest diameter (fig. 8).

At 228 hours, practically all of the nonfollicular spaces contain blood elements. These spaces are enlarged in size, and show more coalescence than previously. The cytoplasm of the thyroid cells is increased and is very sensitive to hematoxylin dye. The nuclei stain lightly with hematoxylin-eosin. The ultimobranchial body is in close apposition to, but remains apart from, the thyroid lobe.

At 240 hours, the thyroid tissue, which later forms follicles, continues to break up (fig. 7). The rearrangement of cells into spherule formation is in progress, and the nuclei of the thyroid cells stain with varying density. The large nuclei stain lightly with hematoxylin, and the small nuclei stain densely. A few cells contain globules of chromophobic colloid.

Nuclei of the thyroid cells at 252 hours are uniform in size, stain lightly, and possess very little chromatic material. All of the nuclei possess one or two nucleoli. The number of cells containing chromophobic colloid globules is increasing.

TABLE I—Means and Standard Deviations of the Thyroid Follicular Diameters of Male and Female Chicken Embryos

No. of embryos	Age (hr.)	Mean follicular diameter	Microns
10	295	12.0	1.9
10	307	13.6	3.5
10	319	14.0	2.5
10	331	13.0	2.4
10	345	13.3	2.0
10	357	16.3	3.7
10	375	17.6	3.1
10	388	18.3	3.2
10	400	19.0	2.5
10	412	20.0	2.5
10	426	21.0	4.7
10	438	22.6	4.8
10	450	23.0	4.3
10	462	21.0	3.8
10	474	23.0	4.1
10	498	21.0	2.7

An occasional primary follicle is noted in the thyroid tissue at 260 hours of development, and prefollicular globules are rather abundant. These globules are composed of chromophobic colloid. The follicular cells are cuboidal in shape, with the nuclei peripherally located. The cells which contain the chromophobic colloid possess nuclei which are slightly bean-shaped. The colloid is located near the bean-shaped nucleus on the side next to the lumen of the follicle to be formed. The cell outlines are indistinct, and the glandular tissue has a syncytial appearance. It is difficult to ascertain whether these droplets of colloid first appear intracellularly or intercellularly. The fact that colloid is found next to the nucleus would suggest that it first appears intracellularly. The primary follicular cavities are formed by secretion and coalescence of the chromophobic colloid which has its origin near the nucleus on the side next to the lumen of the follicle. Most of the follicles at

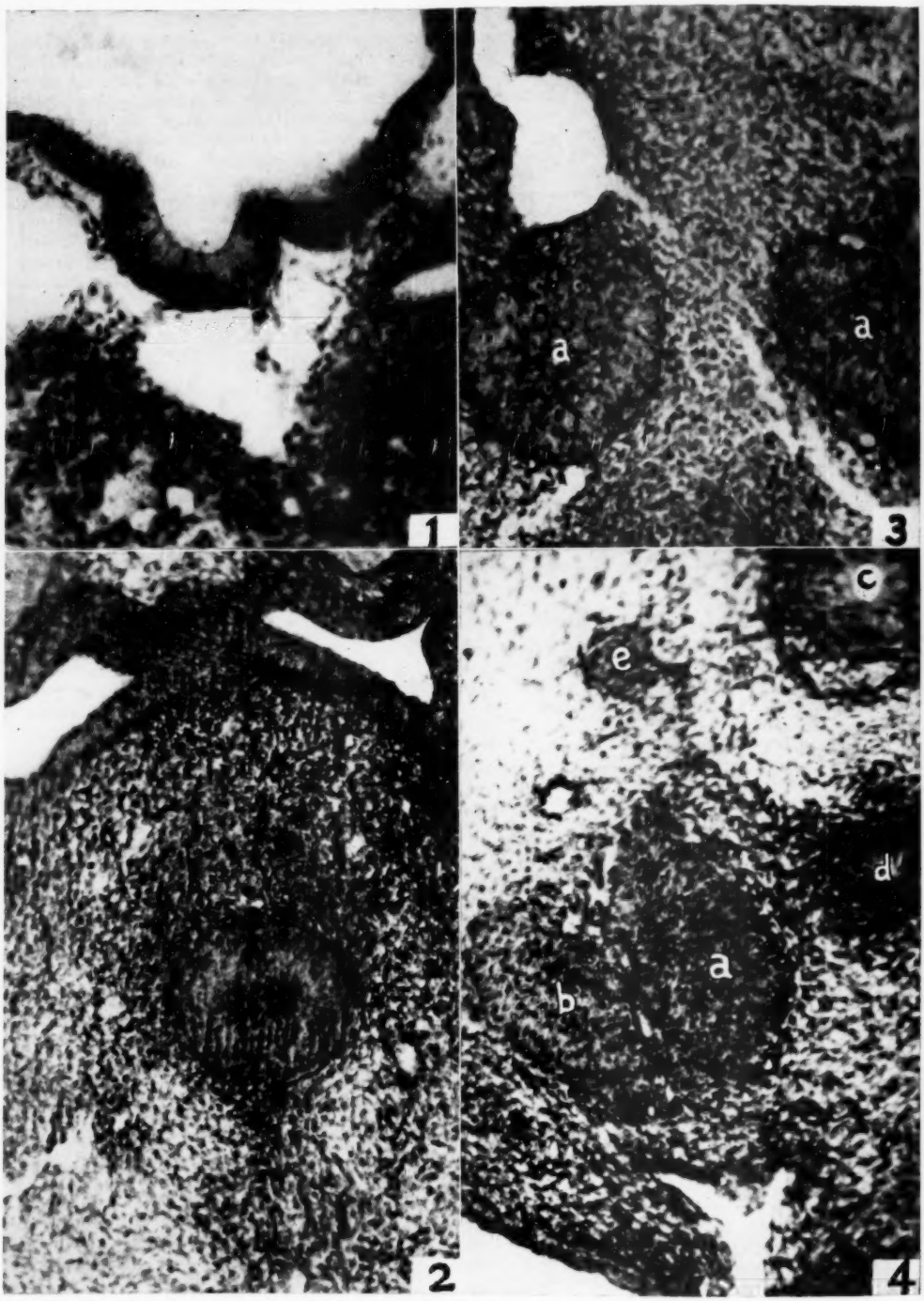

PLATE I

this time are composed of from six to seven cells.

At 272 hours, there are from two to four follicles per section which contain chromophobic colloid (fig. 8, 9). The nuclei of some of the cells are elongated. The cytoplasm of the cells is slightly acidophilic.

At 284 hours, the thyroid tissue has become very compact, and the nonglandular spaces have decreased. This may be caused by the increase in glandular cells following excessive mitotic activity. The colloid in the follicles stains chromophobic to chromophilic.

Follicles typical of those found in the adult thyroid are observed for the first time at 295 hours (fig. 10). The large follicles contain chromophilic colloid, and this colloid may, in turn, contain chromophobic colloid droplets. The follicles vary in diameter from 9 to 16 μ (table 1), measured from basement membrane to basement membrane. Most of the follicles are made up of from six to ten cells. The smaller follicles contain chromophobic colloid. Occasionally, it seems that some cells in a follicle are secreting chromophobic colloid, while others secrete chromophilic colloid.

The thyroid gland at 307 hours shows many follicles whose diameters range from 11 to 16 μ (table 1). At 319 hours, the follicles have a diameter of from 12 to 14 μ, and the largest follicles do not measure more than 20 μ in diameter. The glandular cells are of two types, those which stain deeply with eosin, and others which stain very lightly with eosin. Those cells which are entering into follicular formation stain deeply and possess small, deeply basophilic nuclei. The lightly staining cells, which possess very large, rather clear nuclei, do not enter into follicular formation. Most of the mitotic activity is occurring among the lightly staining cells. These cells are to be interfollicular cells.

The follicles in the thyroid at 352 hours are more numerous and, as a whole, have increased slightly in size. The largest follicles do not excede 25 μ, and there are many between 15 and 20 μ. Most of the follicles contain both types of colloid. Many follicular epithelial cells present chromophobic colloid in their cytoplasm next to the nucleus on the side toward the follicular lumen. Vascular spaces surround part of every follicle. In some cases, thymic tissue bounds the lateral surface of the thyroid glands.

At 375 hours, the largest follicle measures 30 μ in diameter (fig. 11). Growth of the follicles is achieved by a fusion of the follicular epithelium with cells which have not yet organized into follicular formation. An increase in the number of follicles is accomplished by follicular formation among the epithelial cords. Nearly all the follicles contain chromophobic colloid droplets. The follicles are all in contact with the vascular spaces. Tubelike openings occur in the follicular wall.

The thyroid follicles at 400 hours average 19 μ in diameter (fig. 13). The largest follicle measures 30 μ in diameter. The average follicular epithelial height is 8 μ. The type of colloid which the follicle contains is not correlated with size. Chromophobic and chromophilic colloids are found in follicles of all sizes; however, the follicles which contain only chromophobic colloid are usually about 15 μ or less in diameter.

The mean follicular diameter at 438 hours is 22.6 μ, and the mean follicular cell height is 8 μ. Many single, tubelike openings proceed from the lumens of follicles to the surrounding circulatory spaces (fig. 13, 14). The basement membrane remains intact, and colloid does not stream out of these openings. Cells which are undergoing mitotic changes are present among the cells of the follicular epithelium.

The mean follicular diameter at 450 hours is 23 μ. The cytoplasm of the follicular epithelial cells is markedly granular. The interfollicular cells are slightly larger than the fol-

Legends for Illustrations on Opposite Page

PLATE I

Fig. 1—A sagittal section taken from a 72-hour chicken embryo. Observe the high columnar cells and the saucer-shaped depression in the epithelium of the floor of the pharynx. This is a beginning stage of thyroid gland development. Hematoxylin-eosin stain. x 200.

Fig. 2—A transverse section taken from a 110-hour chicken embryo. The thyroid primordium has lost connection with the pharyngeal epithelium and is a spherical mass of cells migrating posteriorly to its intrathoracic position. Hematoxylin-eosin stain. x 200.

Fig. 3—A sagittal section taken from a 120-hour chicken embryo. The thyroid primordium has just become bilobed; (a) thyroid lobes. Hematoxylin-eosin stain. x 200.

Fig. 4—A sagittal section taken from a 148-hour chicken embryo. On the posterolateral surface of the thyroid lobe, a group of cells are being detached and will become the anterior lobe of the parathyroid gland. The parathyroid primordium becomes detached from the thyroid lobe as a result of mesenchymal connective tissue invasion into the thyroid tissue; (a) thyroid lobe; (b) parathyroid primordium; (c) esophagus; (d) trachea; (e) thymic primordium. Hematoxylin-eosin stain. x 200.

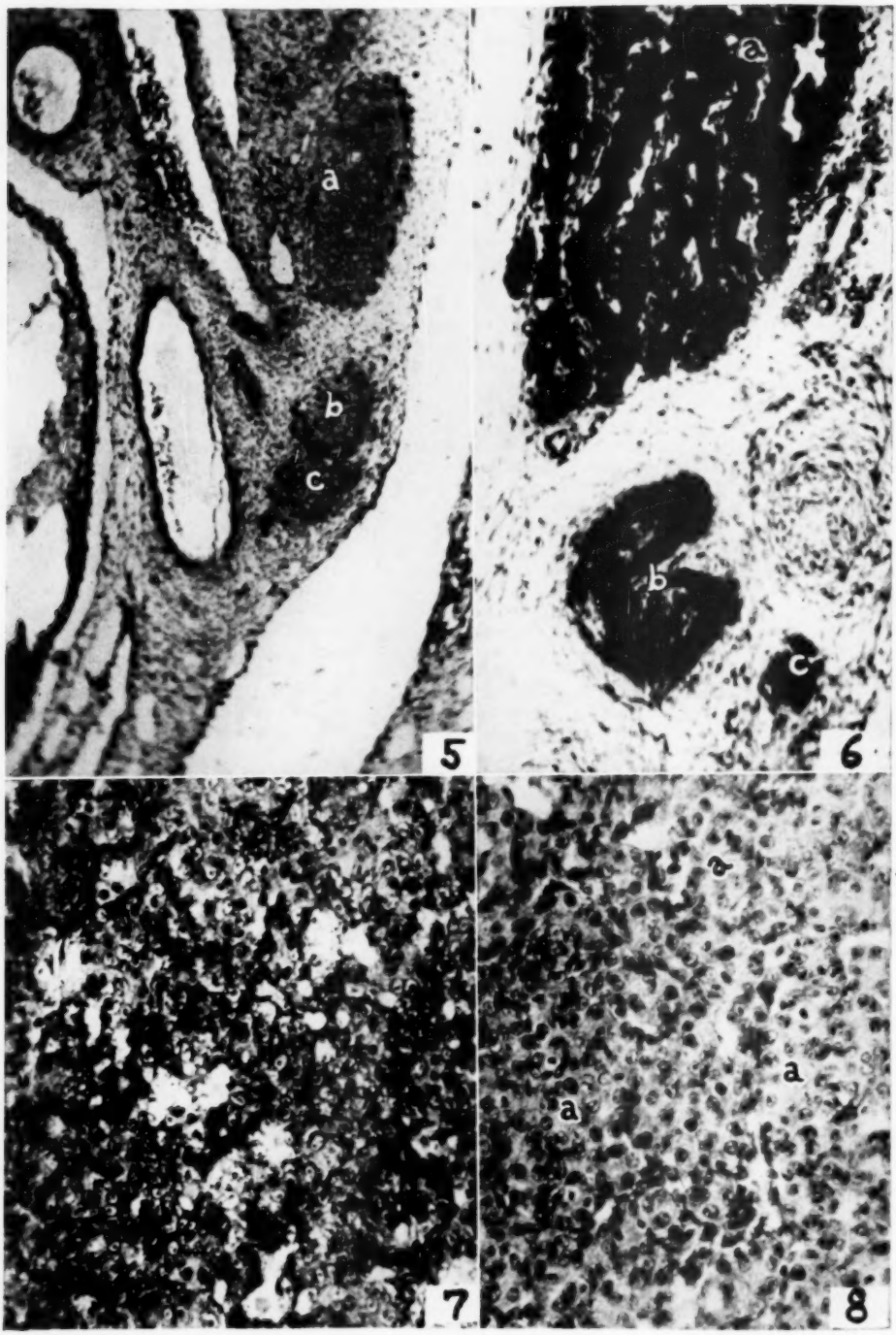

PLATE II

licular cells. Their nuclei stain lightly basophilic, and the cytoplasm is rather clear and without granules. The glandular tissue is very compact and has crowded many of the vascular spaces out of existence. The endothelial lining of these spaces remains between the follicles, forming a basement membrane for the follicular cells. The largest vascular spaces are found at the periphery of the gland. Many of these spaces still contain erythrocytes. The connective tissue capsule surrounding the gland is increasing in thickness, and is sending fine trabeculae into the parenchyma.

The mean follicular diameter at 474 hours is 23 μ, and the mean follicular cell height is 7 μ (fig. 12). Droplets of chromophobic colloid are present in many of the follicles, giving the colloid a vacuolated appearance.

The mean follicular diameter at 498 hours is 21 μ, and the mean follicular epithelial height is 6.5 μ. The glands are very compact, reducing the size of the remaining vascular spaces. The interfollicular cells are larger than the follicular epithelial cells; they range between 8 and 9 μ in diameter. Follicles containing only chromophobic colloid, and those containing chromophilic colloid and large droplets of chromophobic colloid are still present.

DISCUSSION

The observation that the entire thyroid primordium of the chick develops from an unpaired median ventral evagination of the pharyngeal epithelium (fig. 1) agrees with the view held by Remak,[6] Mall,[4] Verdun,[12] and Marine.[5] This evagination occurs in the region of the first branchial cleft; as development proceeds, the thyroid primordium migrates posteriorly with the aortic arches to a final intrathoracic position. During early development, the thyroglossal duct and isthmus disappear. This view of the development of the thyroid does not agree with that held by Stiedo,[7] who stated that the gland has a lateral, as well as a median, origin. Mall[4] designated the derivatives of the fourth visceral pouch as "Korpern Y", and concluded that they do not participate in the development of the thyroid. The present study agrees with Mall's conclusion.

The age at which the thyroid primordium first appears varies among the embryos examined. All of the embryos show thyroid primordium at 40 hours of development. This observation is supported by the reports of Lillie,[3] Yoshikawa,[13] and Bradway.[1]

The thyroid primordium loses connection with the pharynx at 96 hours of development. This observation is in agreement with that of Lillie.[3] Yoshikawa[13] found that the thyroid anlage loses connection with the pharyngeal epithelium by 72 hours; this appears to be much too early. On the other hand, Bradway[1] observed that the thyroid anlage loses pharyngeal connection at 120 hours. This seems to be very late, and does not agree with the above observation that the gland becomes completely paired at 120 hours.

The first indication of pairing of the thyroid gland occurs at 110 hours of incubation (fig. 2); complete pairing takes place at 120 hours (fig. 3). This observation agrees exactly with that of Bradway[1] and of Yoshikawa.[13] The thyroid anlage loses connection with the pharyngeal epithelium prior to pairing. This observation does not agree with that of Bradway,[1] who found loss of pharyngeal connection and pairing occurring about the same time. Verdun[12] stated that complete pairing of the gland does not occur before 172 hours; this is in agreement with Lillie,[3] who found that complete pairing occurs at 168 hours of incubation. The above observations do not agree with these authors.

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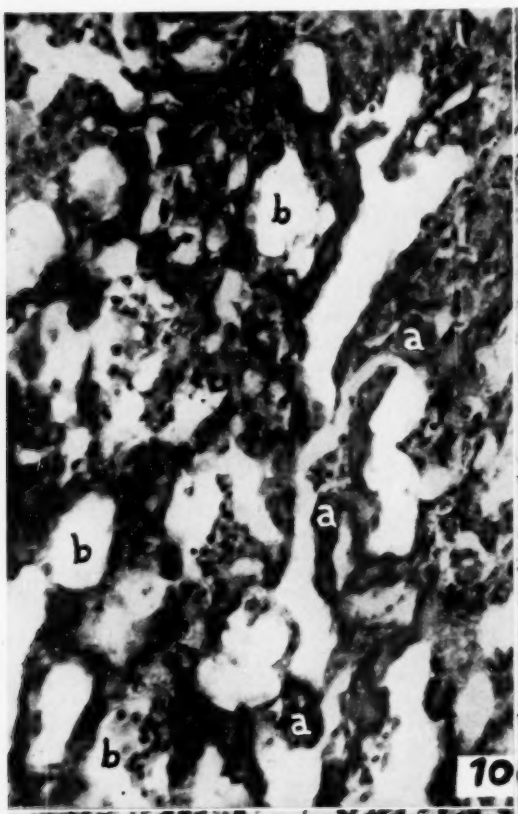
PLATE II

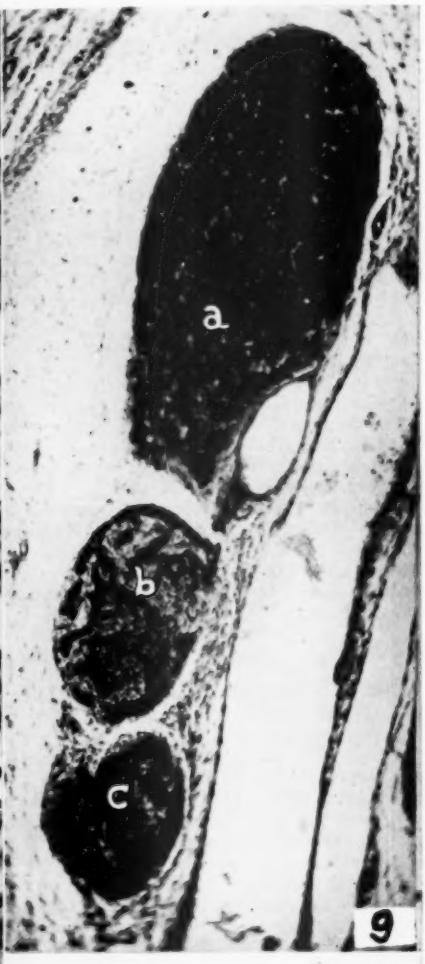
Fig. 5—A sagittal section taken from a 165-hour chicken embryo. At this time, the parathyroid primordium has migrated posteriorly from the thyroid lobe and is detaching a group of cells which become the small posterior lobe of the parathyroid gland; (a) thyroid lobe; (b) parathyroid primordium which will become the anterior lobe; (c) parathyroid primordium which will become the posterior lobe. Hematoxylin-eosin stain. x 100.

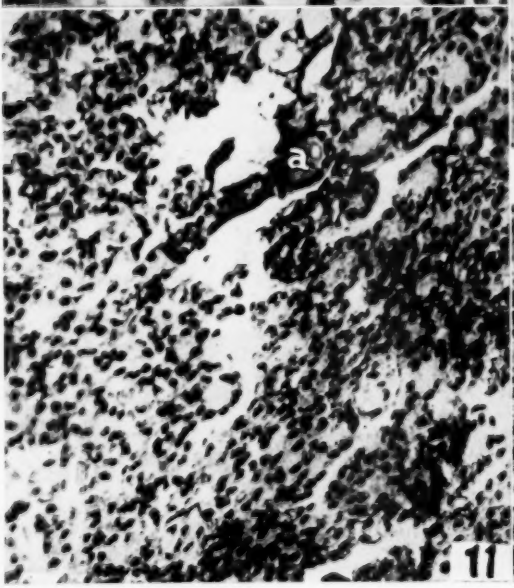
Fig. 6—A sagittal section taken from a 213-hour chicken embryo. Observe the cordlike arrangement of the thyroid tissue and the nonfollicular spaces between the epithelial cords; (a) thyroid lobe; (b) anterior lobe of parathyroid gland; (c) posterior lobe of parathyroid gland. Hematoxylin-eosin stain. x 200.

Fig. 7—A section of thyroid tissue taken from a 240-hour chicken embryo. Careful examination will reveal clear cytoplasm about some of the epithelial cell nuclei. These clear cytoplasmic areas contain chromophobic colloid. Hematoxylin-eosin stain. x 400.

Fig. 8—A section of thyroid tissue taken from a 272-hour chicken embryo. Observe the clear cytoplasmic areas about some of the nuclei; (a) follicles containing chromophobic colloid. Hematoxylin-eosin stain. x 400.







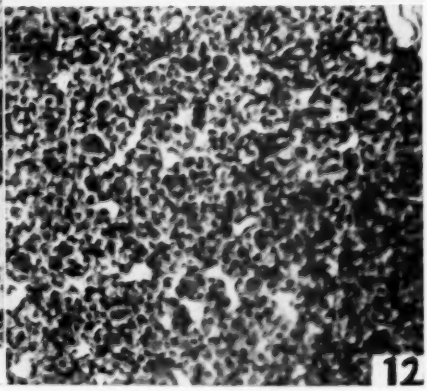


PLATE III

Clearly defined, nonfollicular spaces are observed at 148 hours. This agrees fairly well with the work of Bradway[1] and Yoshikawa,[13] who stated that the thyroid gland acquires nonfollicular cavities at 6 days, and at 5¼ days, respectively. It was observed that at 165 hours the plates of epithelial cells, separated by nonfollicular spaces, become distinctly visible (fig. 5, 6). Slight variation exists between the results of Bradway,[1] who stated that epithelial cell plates become visible at 8 days; of Yoshikawa,[13] who found epithelial plates formed between 5½ and 11 days; and of Sun,[8] who reported visible epithelial plates at 6 days.

Intracellular chromophobic colloid appears at 240 hours (fig. 7, 8), and the first primary follicle at 260 hours. Bradway[1] observed colloid for the first time at 11 days, and follicular formation at 13 days. This observation agrees with that of Yoshikawa,[13] who observed colloid at 11½ days. Hopkins[2] found chromophilic colloid in the glands of 10-day-old embryos. Physiological studies by Sun[8] indicated that the active principle of the thyroid is detectable in the thyroid at ten days, and thereafter increases in both relative and absolute amounts.

The first colloid secreted is chromophobic in nature (fig. 10). This agrees with the conclusions of Bradway,[1] and Yoshikawa.[13] Hopkins,[2] on the contrary, stated that chromophilic, rather than chromophobic, colloid is first formed. All investigators agree that the colloid is first observed intracellularly. The colloid in the follicles of the 284-hour thyroid gland has a tendency to stain chromophilic, but does not become definitely chromophilic until 295 hours (fig. 11). Bradway[1] found that the colloid of the thyroid of the 12-day-old chicks, which had previously been chromophobic, is chromophilic.

The primary follicular cavities are formed by secretion and coalescence of the chromophobic colloid, which has its origin near the nucleus of the follicular cells on the side next to the future lumen of the follicle. Growth of the follicles is achieved by a fusion of the follicular epithelium with cells which have not yet organized into follicular formation. Occasionally, a follicular epithelial cell is found in a state of mitosis; this may account for some of the follicular growth. Bradway[1] is of the opinion that follicles become larger by proliferation of follicular cells and consequent addition to the stored colloid. Further, Bradway found that growth follicles by fusion of follicles and multiplication by budding takes place in very rare instances. Yoshikawa[13] observed that interfollicular epithelial cells form new follicles.

Different cells in the same follicle secrete either chromophobic or chromophilic colloid at the same time. This observation was first made at 295 hours. Bradway[1] made a similar observation. No cells were found to contain both chromophobic and chromophilic colloid at the same time, as was noted by Hopkins.[2]

When chromophobic colloid is present in a follicle which contains chromophilic colloid, the follicular colloid gives the impression that vacuoles are present (fig. 14). Therefore, the so-called vacuoles which have been described in the thyroids of various animals are probably chromophobic colloid. Because of the increased secretion of droplets of chromophobic colloid in the late embryonic stages, it is thought that the function of this colloid is to dilute the more dense chromophilic colloid.

The method by which the follicular contents reach the circulatory system is controversial. Hopkins[2] described definite, single, tubelike openings leading from the lumens of follicles to the surrounding circulatory spaces. She believed that such an opening is functional during each functional cycle of a follicle, and thereby furnishes a route by which the colloid reaches the circulatory system. The evidence presented by this investigation shows that these openings are not true openings (fig. 13). Upon careful examination, it can be seen that the

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PLATE III

Fig. 9—A sagittal section taken from a 272-hour chicken embryo. Observe the relations of the thyroid and parathyroid lobes; (a) thyroid lobe; (b) anterior lobe of parathyroid; (c) posterior lobe of parathyroid. Hematoxylin-eosin stain. x 100.

Fig. 10—A section of thyroid tissue taken from a 295-hour chicken embryo; (a) primary follicles; (b) vascular spaces. Hematoxylin-eosin stain. x 400.

Fig. 11—A section of thyroid tissue taken from a 375-hour chicken embryo; (a) primary follicles containing chromophilic and chromophobic colloid. Hematoxylin-eosin stain. x 400.

Fig. 12—A section of thyroid tissue taken from a 474-hour chicken embryo. Hematoxylin-eosin stain. x 100.

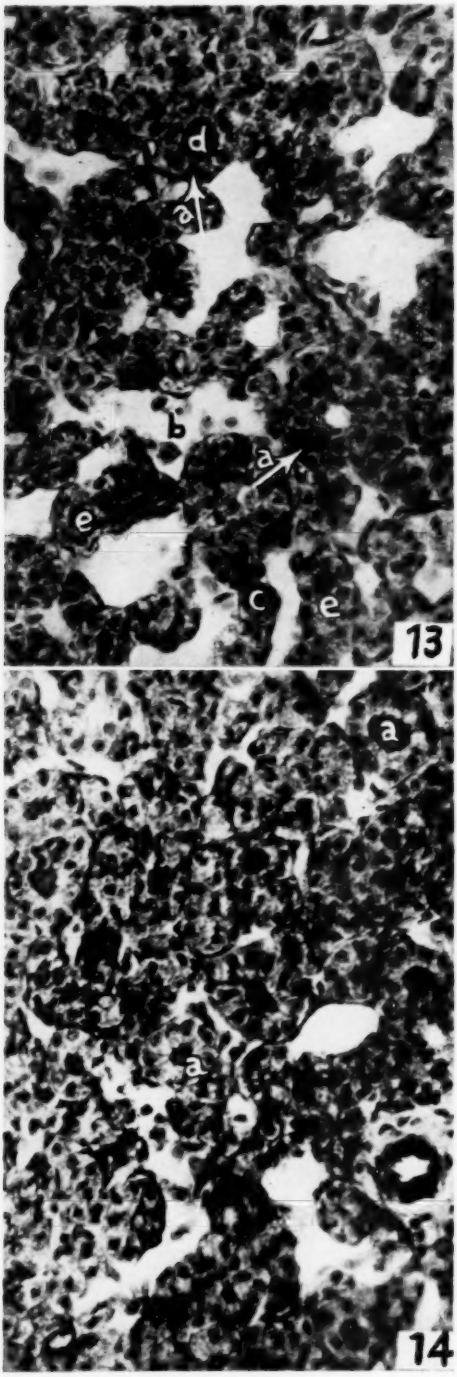

PLATE IV

tubelike openings are actually cells filled with colloid. The surface of the cell nearest the lumen of the follicle is not intact. The cell, however, is not degenerating, since the nucleus appears normal as compared with other follicular cell nuclei. As a result, colloid seems to push through the cytoplasm to the base of the cell, which usually borders a vascular space. The appearance, at first glance, is that of colloid passing directly from the lumen of the follicle to a vascular space. There is, however, a basement membrane which prevents the passage of the colloid directly into the vascular system. In no case was there an appearance of colloid streaming into a vascular space. Therefore, it is believed that the active principle of the thyroid follicle reaches the circulatory system by physicochemical processes through the basement membrane, which is formed from the endothelial lining of the vascular spaces.

The follicular epithelial height was found to vary only slightly during the developmental stages of the chick. The follicular diameters show some significant variability. The maximum diameter attained during embryonic development, for a single follicle, was 30 μ. There is a progressive increase in the mean follicular diameter from 295 hours to hatching (fig. 8, 10, 11, 12, 13, 14).

No sex differences occur between the percentage weights of the thyroid glands from the tenth to the twenty-first day.[10] The possible functional interrelationship of the thyroid cells and the other endocrine glands, based on morphology of the developing chicken embryo, has previously been reported.[11]

SUMMARY

The thyroid gland begins development from an unpaired median ventral evagination of the pharyngeal epithelium at about 36 hours of incubation.

The thyroid anlage loses connection with the pharynx at 96 hours and becomes bilobed by 120 hours of incubation.

PLATE IV

Fig. 13—A section of thyroid tissue taken from a 400-hour chicken embryo. Several of the follicles appear to have tubelike openings into the surrounding vascular space. These openings, however, are not true openings, but rather follicular epithelial cells filled with chromophilic colloid. Note the simple squamous epithelium lining the vascular spaces, and its relation to the follicle; (a) arrow points to tubelike opening; (b) vascular space; (c) follicle containing chromophobic colloid; (d) follicle containing chromophilic colloid; (e) follicle containing both chromophobic and chromophilic colloid. Hematoxylin-eosin stain. x 400.

Fig. 14—A section from the thyroid gland of a 438-hour chicken embryo; (a) follicle containing both chromophobic and chromophilic colloid. Hematoxylin-eosin stain. x 400.

Nonfollicular spaces appear in the thyroid glands at 148 hours, and distinct epithelial cell plates appear at 165 hours of development.

Intracellular chromophobic colloid first appears at 240 hours, and the first primary follicles at 260 hours. Chromophilic colloid appears at 295 hours of incubation, at which time the follicles are typical of those present in the adult thyroid.

Primary follicular cavities are formed by secretion and coalescence of the chromophobic colloid which has its origin near the nucleus on the side of the cell next to the future lumen of the follicle. Growth of follicles is achieved by fusion of the follicular epithelium with cells which have not yet organized into follicular formation, and by follicular cell division.

Since the follicular cells lining a follicle may secrete either chromophobic or chromophilic colloid, a follicle contains two types of colloid. Chromophobic colloid mixed with chromophilic colloid gives the follicular colloid a vacuolated appearance. Therefore, it is concluded that the previously described vacuoles in the follicular colloid are not true vacuoles.

The follicular epithelial cell height varies only slightly during embryonic life.

There is a progressive increase in the mean follicular diameters from 295 hours of incubation until hatching.

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The Binding of Sulfonamide Drugs to the Plasma Proteins of Chicken Blood

R. A. BANKOWSKI, D.V.M., Ph.D., and RUTH E. JOHNSON, A.B.

Berkeley, California

DAVIS[1] AND DAVIS AND WOOD[2] reported that a positive quantitative correlation was obtained *in vitro* between the bacteriostatic power of some of the commonly employed sulfonamides and their protein-binding tendencies for the albumin of blood plasma of man. They have also demonstrated that the bacteriostatic activity of these drugs was dependent upon the molecular structure of the sulfonamides and their ionic dissociation. Further evidence showed that the bound fraction of the sulfonamide was bacteriostatically inactive.

The pharmacodynamics of some sulfonamides has been observed to differ, in some respects, in chickens as compared to other species of animals. It was of interest to ascertain, therefore, whether chicken plasma proteins would bind the sulfonamides to the same degree as has been reported for the plasma proteins of man.

METHODS

The method described by Davis[3] for determining the binding of drug to plasma proteins was used. It consisted essentially of dialyzing plasma, at refrigerator temperature, in a cellophane bag against a buffered solution at pH 7.4 containing the sulfonamide. The period of dialysis was maintained for sixty-four hours. The amount of drug bound to protein was calculated from the relative concentrations of the sulfonamide on either side of the membrane.

The plasma was obtained from pooled blood samples, collected by cardiac puncture from 2 to 6 apparently normal chickens of the same age group. Heparin was used as an anticoagulant. Several lots of plasma were used in the tests, and the protein concentration of each lot of serum was determined at the end of the dialysis period by the micro-Kjeldahl technique. Inasmuch as the extent of sulfonamide binding was expressed in per cent of bound drug per gram of plasma protein, individual variations due to differences in protein concentrations were not materially affected. No correction for the Donnan effect was made.[4] Sulfonamide determinations were made by the method of Marshall and Bratton,[5] using a photoelectric colorimeter to measure the color reactions.

From the Department of Veterinary Science, University of California, Berkeley.

RESULTS

The affinities of the commonly employed sulfonamides for the plasma proteins of chicken blood, expressed in a ratio of bound to free drug in per cent per gram of plasma protein, are summarized in table 1. Sulfanilamide and sulfaguanidine showed the least tendency, of the group of drugs tested, to bind the plasma proteins. The relative protein-binding capacity of sulfamerazine, sulfamethazine, and sulfathiazole increased in the order named. The greatest affinity for the plasma proteins in chicken blood was demonstrated by sulfaquinoxaline. Schlenker[6] reported incomplete recoveries of sulfaquinoxaline from whole blood and protein-containing solutions. The great affinity of plasma proteins for this sulfonamide, especially when fresh samples of plasma were employed, may explain the difficulty in obtaining complete removal of sulfaquinoxaline from the proteins.

In addition to the factors which influence sulfonamide binding by the proteins noted by Davis,[3] results obtained indicate the importance of age of the plasma in relation to its binding tendency. This was especially evident with sulfaquinoxaline, whose binding capacity decreased with the age of the plasma sample employed (table 1).

Knowledge concerning the relative bacteriostatic activity of the various sulfonamides used against bacterial infections in chickens is meager. Therefore, a correlation between therapeusis of a sulfonamide toward bacterial infections of this species can not be made at this time. On the other hand, the anticoccidial action of the sulfonamides has been repeatedly tested and a rough correlation between the coccidiostatic effect of the sulfonamides (except sulfathiazole) and their binding tendencies to plasma proteins exists.

SUMMARY

The plasma proteins of chicken blood bind the commonly employed sulfonamides in the following increasing order: sulfaguanidine, sulfanilamide, sulfamerazine,

TABLE I—Binding of Six Sulfonamides to the Plasma Proteins of Chicken Blood

	Plasma		Drug concentration		Plasma protein (Gm. %)	Ratio of bound to free drug expressed in %/Gm. plasma protein
	Age and sex of chicken	Age of plasma	Buffer M x 10^{-4}	Plasma M x 10^{-4}		
Sulfaguanidine	adult ♂ + ♀	24 hr.	2.24	2.57	3.66	4.8
	40 da.	4 da.	4.11	4.58	3.84	3.7
	40 da.	4 da.	4.18	4.63	3.96	3.5
	40 da.	4 da.	4.14	4.49	3.63	3.0
	adult ♂ + ♀	24 hr.	4.60	4.91	3.72	2.6
Sulfanilamide	adult ♂ + ♀	4 da.	2.62	3.11	4.43	5.0
	adult ♂ + ♀	4 da.	5.15	6.05	4.31	4.8
	adult ♂ + ♀	4 da.	7.67	8.87	4.29	4.4
Sulfamerazine	adult ♂ + ♀	4 da.	1.71	2.35	4.56	8.97
	adult ♂ + ♀	4 da.	3.41	4.33	4.37	6.90
	adult ♂ + ♀	4 da.	5.26	6.66	4.29	6.95
Sulfamethazine	adult ♂ + ♀	24 hr.	1.50	2.31	3.99	14.2
	40 da.	4 da.	2.87	4.33	3.63	14.8
	40 da.	4 da.	2.89	4.30	3.73	14.0
	40 da.	4 da.	2.89	4.40	3.59	15.3
	adult ♂ + ♀	24 hr.	3.03	4.48	4.02	12.6
Sulfathiazole	adult ♂ + ♀	4 da.	1.68	3.45	4.18	23.4
	adult ♂ + ♀	24 hr.	1.86	4.58	3.79	39.3
	adult ♂ + ♀	4 da.	3.34	6.41	4.06	23.4
	adult ♂ + ♀	4 da.	4.93	8.69	4.04	19.6
Sulfaquinoxaline	adult ♂ + ♀	4 da.	1.02	2.55	4.17	36.7
	6 mo. ♂ + ♀	immed.	2.05	14.90	3.77	168.0
	adult ♂ + ♀	4 da.	2.49	5.11	4.27	25.4
	adult ♂ + ♀	24 hr.	2.40	12.50	3.89	110.0
	adult ♂ + ♀	4 da.	3.80	7.24	4.00	23.3
	6 mo. ♂ + ♀	immed.	6.20	25.05	3.80	78.9
	6 mo. ♂ + ♀	immed.	10.95	35.00	3.77	58.9

sulfamethazine, sulfathiazole, and sulfaquinoxaline.

The binding capacity of the plasma proteins of chicken blood, especially noted with sulfaquinoxaline, appeared to decrease with the age of the plasma sample employed.

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Studies on Certain Filterable Viruses
X. Immunogenic Properties of *Borreliota Avium* Suspended in Buffered Glycerol and in Mineral Oil

H. S. BRYAN, D.V.M., M.S.

Urbana, Illinois

Borreliota avium (fowlpox) vaccines have been prepared from various virus-containing tissues. Virus originating from skin-lesion material, chorioallantoic membranes of inoculated eggs, and from the entire embryo has been used. Woodruff and Goodpasture[7] introduced bacteria-free chorioallantois-propagated virus. Brandly[1,2] and Dunlap[4] showed that this source of the virus could be successfully used in immunizing healthy, susceptible chickens. The use of the entire developing chicken embryo, consisting of a composite of chorioallantois, yolk, yolk sac, albumen, embryo proper, and fluids from inoculated embryos was reported by Thorning, Graham, and Levine,[6] and Kerlin and Graham.[5]

The desiccated form of *Bor. avium*, whether in skin lesion material, chorioallantois, or the entire embryo, is the source of the virus for commercial vaccine production at present. Preliminary observations suggested that a vaccine might be made from undesiccated entire embryo virus suspended in suitable diluents. The present paper records the preparation, viability, and immunogenic properties of undesiccated entire embryo fowlpox virus suspended in 50 per cent buffered glycerol pH 7.6 and in mineral oil.

Materials and Methods

Preparation of Virus and Vaccines.—The fowlpox virus employed in these studies was a single strain originating in a field outbreak of fowlpox in chickens in Illinois. Since its isolation, the virus had been maintained by serial passage on the chorioallantoic membrane of developing chicken embryos. For storage, the virus-bearing tissues were desiccated and held at 5 C. Eggs showing actively developing, vigorous embryos were inoculated after ten to eleven days' preliminary incubation. Inoculations were made by a modification of Burnet's[3] technique. After five days' further incubation, the eggs were examined for evidence of virus multiplication. Those membranes showing characteristic fowlpox lesions on the chorioallantois were harvested aseptically by removing the contents of the egg to sterile Petri dishes. Following harvest, the entire embryos were transferred to an electric tissue grinder and homogenized to the consistency of thick cream. The homogenized embryos were placed in sterile flasks and either used immediately in vaccine preparation or stored at –5 C. until needed.

Heavy white mineral oil (liquid petrolatum) U.S.P. and 50 per cent buffered glycerol were the only suspending agents used in these studies. The glycerol diluent was prepared by dissolving 375 cc. of glycerol (C.P.) and 1.62 Gm. of anhydrous potassium dihydrogen phosphate in 375 cc. of distilled water. Sodium hydroxide was used to adjust the pH to 7.6 prior to sterilization. Both the mineral oil and buffered glycerol diluents were sterilized for forty-five minutes under 15 lb. of pressure.

To determine the duration of viability of undesiccated entire embryo *Bor. avium*, suspensions of the virus were prepared in 50 per cent buffered glycerol and in mineral oil. Only one lot of undesiccated entire embryo virus was used in preparing the various fowlpox vaccines employed in the viability tests. Seven vaccines were prepared as follows: (1) entire embryo virus alone; (2) 10 per cent entire embryo virus in mineral oil; (3) 5 per cent entire embryo virus in mineral oil; (4) 1 per cent entire embryo virus in mineral oil; (5) 10 per cent entire embryo virus in 50 per cent buffered glycerol; (6) 5 per cent entire embryo virus in 50 per cent buffered glycerol, and (7) 1 per cent entire embryo virus in 50 per cent buffered glycerol. Each vaccine was then divided into three equal portions for storage at temperatures of 14 C., 5 C., and –5 C. Thus, a total of 21 vaccines was obtained. At the time of preparation, and at two- or three-month intervals thereafter, the vaccine preparations stored at each of the three temperatures were tested by inoculation of fowlpox-susceptible chickens. Viability tests were continued in most cases until no virus response was elicited.

Three-week-old White Leghorn or Rhode Island Red chickens, known to be susceptible to fowlpox, were routinely used in making viability tests on the experimental vaccines. The three-stick method of inoculation into the wing patagium was used. Observations for "take" reactions were made on the seventh, four-

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teenth, and twenty-first days after inoculation. To determine the protection produced by inoculation with the various vaccine preparations, the experimental birds were exposed to a homologous strain of entire embryo virus twenty-one days after the original inoculation. The feather-follicle method on the thigh was used for this purpose. As a check on the potency of the exposing virus, a small number of control chickens was also inoculated with the exposing virus simultaneously with the other chickens. Observations for take reactions were made on the seventh and fourteenth days after reëxposure. A total of 507 chickens was employed.

Buffered Glycerol and Mineral Oil Diluents.—To determine the duration of viability of undesiccated entire embryo *Bor. avium*, suspensions of the virus in 50 per cent buffered glycerol pH. 7.6 and in mineral oil were prepared by the method described above.

As will be noted in table 1, the experimental fowlpox vaccines stored at 5 C. and at –5 C. proved viable longer than those stored at 14 C. Each vaccine was viable at the time of preparation and produced a typical fowlpox reaction at the site of inoculation.

At the 14 C. storage temperature, the entire embryo virus alone and 10 per cent, 5 per cent, and 1 per cent entire embryo virus in mineral oil proved viable for two months. These were nonviable at five months. The 5 per cent entire embryo virus suspended in 50 per cent buffered glycerol remained viable longer than any of the other vaccine preparations stored at this temperature. It was found nonviable after ten months' storage.

Of the vaccines stored at 5 C., those containing 50 per cent buffered glycerol as the diluent proved superior to those prepared with mineral oil. Entire embryo virus alone and 10 per cent and 5 per cent entire embryo virus in mineral oil were viable for fourteen months but nonviable at seventeen months. The vaccine containing 1 per cent entire embryo virus in mineral oil was nonviable at ten months. One per cent entire embryo virus in 50 per cent buffered glycerol was viable at fourteen months but nonviable at seventeen months. Ten per cent and 5 per cent entire embryo virus in 50 per cent buffered glycerol were still viable at twenty-five months. Further tests were not made on these two vaccines.

Viability test results of the vaccines stored at –5 C. did not differ markedly from those reported for the vaccines stored at 5 C. Significantly, 10 per cent and 5 per cent entire embryo virus in 50 per cent buffered glycerol were viable for twenty-five and twenty-one months, respectively. Further tests on the former vaccine were not made.

Results of this experiment indicated that the

TABLE I—Survival of Borreliota Avium

No.	Vaccine	Storage Temperature	Survival in Months after Preparation 0 2 5 8 10 12 14 17 19 21 23 25
1.	Entire embryo virus alone	14 C.	——
2.	10% EE in mineral oil	14 C.	——
3.	5% EE in mineral oil	14 C.	——
4.	1% EE in mineral oil	14 C.	——
5.	10% EE in 50% buffered glycerol	14 C.	——
6.	5% EE in 50% buffered glycerol	14 C.	———
7.	1% EE in 50% buffered glycerol	14 C.	———
8.	Entire embryo virus alone	5 C.	————————
9.	10% EE in mineral oil	5 C.	————————
10.	5% EE in mineral oil	5 C.	————————
11.	1% EE in mineral oil	5 C.	———
12.	10% EE in 50% buffered glycerol	5 C.	———————————+
13.	5% EE in 50% buffered glycerol	5 C.	———————————+
14.	1% EE in 50% buffered glycerol	5 C.	————————
15.	Entire embryo virus alone	5 C.	————————
16.	10% EE in mineral oil	5 C.	————————————
17.	5% EE in mineral oil	5 C.	————————
18.	1% EE in mineral oil	5 C.	———
19.	10% EE in 50% buffered glycerol	5 C.	————————————
20.	5% EE in 50% buffered glycerol	5 C.	————————————
21.	1% EE in 50% buffered glycerol	5 C.	————————————

EE = entire embryo virus. 0 = time of preparation. + = viable virus present at end of observation period.

viability of suspensions of undesiccated entire embryo *Bor. avium* depended largely on the storage temperature, and to a lesser degree on the diluent used when mineral oil and 50 per cent buffered glycerol were employed as diluents. It was also noted that the vaccines made up in mineral oil separated out upon storage; those made up in buffered glycerol did not.

Vaccination of Farm Flocks.—Since encouraging laboratory results were being obtained with a 5 per cent suspension of undesiccated entire embryo *Bor. avium* in 50 per cent buffered glycerol stored at 5 C., it appeared worthwhile to use this vaccine in the inoculation of farm flocks of chickens. This vaccine preparation also had other desirable qualities such as ease of preparation, application, and miscibility. Vaccinations were made during the spring and summer months of 1945, 1946, and 1947.

The virus used in the preparation of vaccines for field use was the same strain as employed in the laboratory investigation. A 5 per cent suspension of undesiccated entire embryo *Bor. avium* was prepared in a 50 per cent buffered glycerol diluent and stored at 5 C.

During each of three years (1945, 1946, and 1947), a single lot of the above vaccine was prepared. At the time of field use, the vaccines had been stored from one to seven months. The viability of each lot of vaccine was tested at the time of preparation and at monthly intervals by inoculation of fowlpox-susceptible chickens in the laboratory. Thus, each vaccine was known to possess the ability of producing take reactions in chickens.

Vaccinations were routinely made by the three-stick method into the wing patagium. In 1946 and 1947, vaccinations were made and take reactions were evaluated by the author and other veterinarians; in 1945, results reported by a number of flock owners were also included. Between the seventh and eleventh days following vaccination, a representative number of birds (usually 10% of the flock) was examined for take reactions. Vaccinated birds for artificial exposure were usually not requested.

In 1945, 25,757 chickens in 19 flocks were vaccinated with an average immunity production of 82.7 per cent, as determined by observation for take reactions in approximately 10 per cent of the birds (*see* table 2). The range varied from 100 per cent take reactions reported in four flocks to 25 and 26 per cent in two flocks. The age of the vaccine when dispensed for field use varied from one to five months. In one flock where inoculations had been made by a flock owner and in which 65 per cent take reactions were reported, 5 birds, which did not show positive reactions seven days after inoculations, were obtained for artificial exposure six weeks after the original inoculation. Upon artificial exposure, 4 of the 5 birds proved susceptible to fowlpox, and excellent takes were observed at the site of reinoculation. It is felt that some of the unsatisfactory results obtained in 1945 could be traced to flock owner vaccination.

TABLE 2—Results of Vaccination of Flocks of Chickens with 5 Per Cent Undesiccated Entire Embryo Borreliota Avium, Suspended in 50 Per Cent Buffered Glycerol at pH 7.6*

Year	Flocks (No.)	Birds vaccinated**	Takes reported** (%)
1945	19	25,757	82.7
1946	10	20,497	99.7
1947	6	20,108	98.7
	35	66,362	92.9***

*Age of vaccine, one to seven months, stored at 5 C.
**Vaccinations and interpretations made by flock owners and veterinarians.
***Weighted average.

A total of 20,497 chickens in ten flocks (table 2) was vaccinated with the experimental fowlpox vaccine in 1946. The age of the vaccine varied from one to six months when dispensed for field use. When approximately 10 per cent of the vaccinated birds were checked for positive reactions, 99.7 per cent of these chickens showed take reactions.

In 1947, 20,108 birds in six flocks were vaccinated (*see* table 2). The age of the vaccine varied from four to seven months. The average immunity produced, as determined by the observation of vaccinated birds for take reactions, was 98.7 per cent.

DISCUSSION

Five per cent undesiccated entire embryo *Bor. avium*, suspended in 50 per cent buffered glycerol and stored from one to seven months at 5 C., produced an average of 92.9 per cent take reactions in 35 flocks under field conditions. These flocks included 66,362 chickens. This percentage compares favorably with averages of 95.58 per cent obtained by Thorning, Graham, and Levine[6] and 96.61 per cent reported by Kerlin and Graham[5] when the entire embryo virus was used in the desiccated form.

From the above observations, it appears that this vaccine can be successfully used for the immunization of farm flocks against fowlpox. However, reasonable precautionary measures for the proper handling of the vaccine must be carried out in order to secure satisfactory results. Since time is saved by not desiccating the material, and because the vaccine can be prepared in a laboratory and dispensed in a single vaccine bottle ready for field use, this fowlpox vac-

cine possesses definite advantages over the present commercial fowlpox vaccines.

SUMMARY

The following observations were made in an investigation of the immunogenic properties of undesiccated entire embryo *Borreliota avium*:

1) Experimental vaccines proved immunogenic in fowlpox-susceptible laboratory and farm chickens. Freshly prepared vaccines, consisting of 10 per cent, 5 per cent, and 1 per cent undesiccated entire embryo virus suspensions in mineral oil, and 10 per cent, 5 per cent, and 1 per cent undesiccated entire embryo virus suspensions in 50 per cent buffered glycerol at pH 7.6 and undiluted virus, all produced excellent take reactions at the site of vaccination.

2) The survival of undesiccated *Bor. avium* suspensions in mineral oil and 50 per cent buffered glycerol when held at 14 C., 5 C., and –5 C. for a 25-month storage period was determined. A 5 per cent suspension of entire embryo virus in 50 per cent buffered glycerol at 14 C. was viable for eight months; at 5 C., for twenty-five months; and at –5 C., for twenty-one months. Survival of virus in mineral oil proved shorter than suspensions in 50 per cent buffered glycerol.

3) Entire embryo virus in 50 per cent buffered glycerol diluent was satisfactory and proved superior to the mineral oil diluent.

4) Vaccination of 66,362 chickens in 35 farm flocks, during 1945, 1946, and 1947, with a 5 per cent suspension of undesiccated entire embryo virus in 50 per cent buffered glycerol, resulted in 92.9 per cent positive take reactions. Of the 40,605 birds inoculated during 1946 and 1947, approximately 99.0 per cent showed positive reactions.

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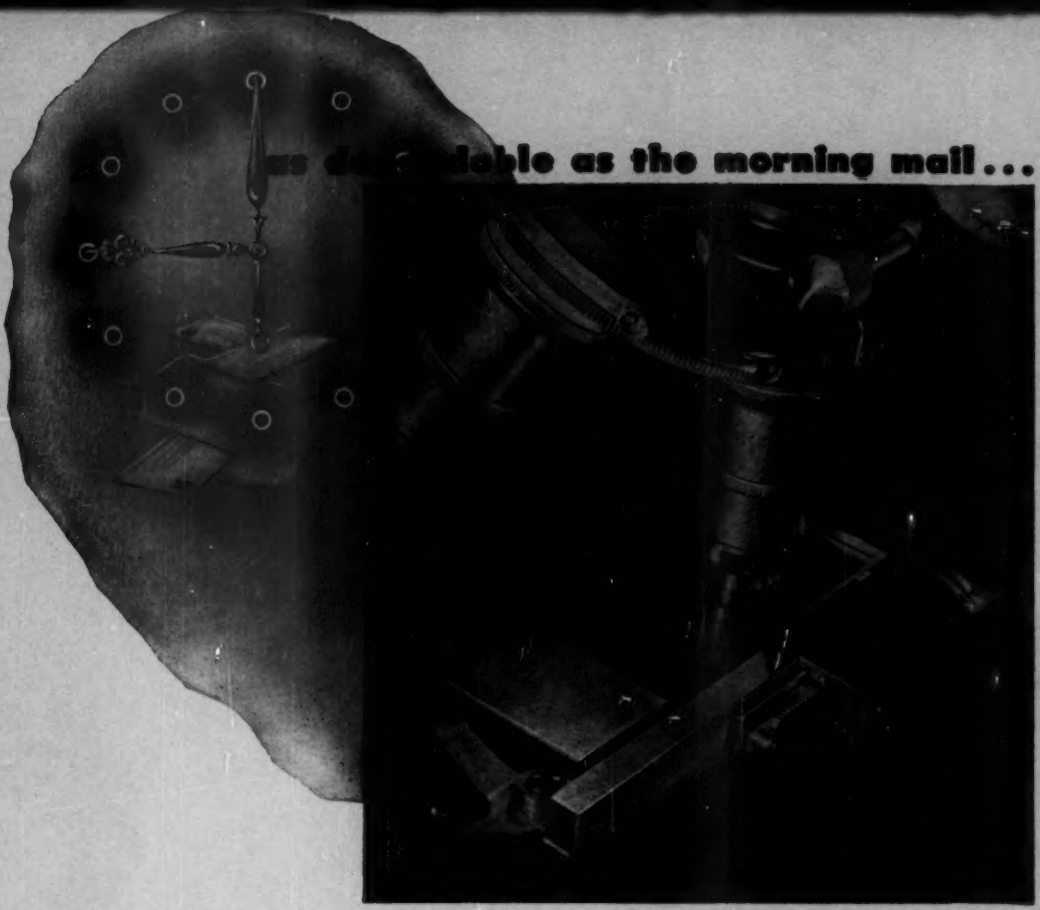

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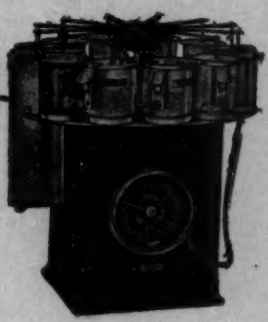

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